



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/82, 5/04, 15/29, 15/05, 15/09, A01H 4/00, 5/10		A1	(11) International Publication Number: WO 99/51758 (43) International Publication Date: 14 October 1999 (14.10.99)
(21) International Application Number:	PCT/US98/27759		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(22) International Filing Date:	24 December 1998 (24.12.98)		
(30) Priority Data:	60/080,852	6 April 1998 (06.04.98)	US
(71) Applicant (for all designated States except US): THE STATE OR OREGON acting by and through THE STATE BOARD OF HIGHER EDUCATION, on behalf of OREGON STATE UNIVERSITY [US/US]; Office of Technology Transfer, 312 Kerr Administrative Building, Corvallis, OR 97331-2140 (US).			Published <i>With international search report.</i>
(72) Inventors; and			
(75) Inventors/Applicants (for US only): MOK, David, W., S. [US/US]; 3024 N.W. Garryanna Drive, Corvallis, OR 97330 (US). MOK, Machteld, C. [US/US]; 3024 N.W. Garryanna Drive, Corvallis, OR 97330 (US). MARTIN, Ruth, C. [US/US]; 3164 N.E. Flintlock, Corvallis, OR 97330 (US).			
(74) Agent: EARP, David, J.; Klarquist, Sparkman, Campbell, Leigh & Whinston, LLP, One World Trade Center, Suite 1600, 121 S.W. Salmon Street, Portland, OR 97204 (US).			

(54) Title: ENZYME RESPONSIBLE FOR THE METABOLISM OF ZEATIN

(57) Abstract

Isolated nucleic acids encoding zeatin O-glucosyltransferase and zeatin O-xylosyltransferase are disclosed. These nucleic acid molecules are useful, among other things, to produce transgenic plants having modified zeatin O-glucosyltransferase activity and/or modified zeatin O-xylosyltransferase activity.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ENZYMES RESPONSIBLE FOR THE METABOLISM OF ZEATIN**FIELD OF THE INVENTION**

5 This invention relates to plant hormones, and in particular to cytokinins. Aspects of the invention include a purified zeatin O-glucosyltransferase enzyme, a purified zeatin O-xylosyltransferase enzyme, nucleic acid molecules encoding these enzymes, and vectors containing all or a portion of the nucleic acid molecules. Transgenic cells and transgenic plants having modified zeatin O-glucosyltransferase and/or modified zeatin O-
10 xylosyltransferase activity are also provided. The invention also relates to altered plant traits in general, and seed development and yield in particular, resulting from the modification of zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase activity in plants.

15 **BACKGROUND OF THE INVENTION**

Cytokinins are plant hormones that mediate cell division and development. This group of hormones was discovered by Miller et al. (1955, 1956) with the identification of the first synthetic cytokinin, kinetin. The first naturally occurring cytokinin, zeatin, was discovered by Letham (1963) in corn and the structure of zeatin was determined by
20 Shaw and Wilson (1964). Zeatin is the most active and ubiquitous cytokinin in all plant species examined. Other naturally occurring cytokinins are structurally related to zeatin (Shaw, 1994).

The critical importance of cytokinins in plant development was illustrated by the classic tissue culture experiments of Skoog et al. (1965). These experiments established
25 that plant cell division requires cytokinin. Furthermore, the ratio of cytokinins to auxins (another group of plant hormones) was shown to determine whether undifferentiated plant cells will develop into shoots (high cytokinin to auxin), or roots (low cytokinin to auxin) or continue to proliferate as callus tissues (intermediate cytokinin to auxin ratio). Thereafter, cytokinin was found to be involved in every phase of plant growth (Mok
30 1994). In general, cytokinins have growth promoting effects, from seed germination and shoot development to retarding senescence and increasing fruit and seed set.

The effects of cytokinins in controlling plant growth have been extensively utilized in plant tissue culture to micropropagate and clone plants and to regenerate whole plants from cells of many species (Krikorian 1995). In fact, the application of
35 cytokinins *in vitro* contributes significantly to advances in plant biotechnology. In agricultural applications, external applications of cytokinins on whole plants are used to obtain enhanced fruit set and grain yield of food crops and longer shelf life of ornamentals (Hradecka and Petr 1992; Karanov et al. 1992; Lewis et al. 1996; Minana

et al. 1989).

Cytokinins are converted to various metabolites in plant tissues (Jameson 1994).

For example, the metabolites of zeatin include O-xylosylzeatin, O-glucosylzeatin, N-glucosylzeatin, zeatin riboside and zeatin nucleotides. The precise functions of these 5 metabolites are still uncertain. However, some of these metabolites may be the stored or the transported form of the active compound, zeatin. The O-glucoside of zeatin may be such a metabolite (Badenoch-Jones 1996). It was first discovered by Letham et al. (1976) and has been found in all crops examined including corn, beans, poplar and soybean. As O-glucosylzeatin can be readily converted back to its active form, zeatin, 10 by the removal of the glucose moiety (via the action of the widespread β -glucosidase enzymes), it is considered a reversible reserve of active cytokinin (Brzobohaty et al. 1993). Also, O-glucosylzeatin is resistant to attack by cytokinin oxidases (McGaw and Horgan 1983) which degrade the parent compound, zeatin. Therefore, O-glucosylzeatin 15 may be important in cytokinin action by serving as a remobilizable reserve and as a resistant form of zeatin.

The enzyme catalyzing the formation of zeatin to O-glucosylzeatin is termed zeatin O-glucosyltransferase. It was first purified and characterized in the inventor's laboratory (Dixon et al. 1989) from lima beans (*Phaseolus lunatus*) as part of a continuing effort to study the metabolism of zeatin (Mok and Martin 1994). The isolation of the 20 enzyme was followed by the generation of specific antibodies that recognize the enzyme (Martin et al. 1990). Enzyme assays and immunoblotting (western) analysis indicated that zeatin O-glucosyltransferase occurs in small developing seeds. As seed size is determined by cell number and cell size, both factors influenced by the level of cytokinins, the occurrence of zeatin O-glucosyltransferase in immature seeds suggests 25 that the enzyme is likely to be of critical importance in determining the eventual seed yield.

Another enzyme that converts zeatin into a metabolite was identified in 1987. This enzyme was termed zeatin O-xylosyltransferase (Turner et al. 1987). The enzyme mediates the formation of O-xylosylzeatin from zeatin using UDPX as the sugar donor. 30 The enzyme has similar properties as zeatin O-glucosyltransferase isolated from *Phaseolus lunatus* but can be distinguished by substrate specificity and charge (Dixon et al. 1989). Briefly, the O-xylosyltransferase utilizes UDPX as the sugar donor while the O-glucosyltransferase uses both UDPG and UDPX but with much greater affinity to UDPG. The O-xylosyltransferase also has higher negative charge than O-xylosyltransferase as 35 determined by anion exchange column chromatography.

Plants having modified endogenous zeatin activity would be of significant agricultural importance. Such plants could be created through genetic engineering if the

genes regulating zeatin were available. It is to such genes that the present invention is directed.

SUMMARY OF THE INVENTION

5 The present invention provides isolated plant nucleic acid molecules (cDNA and genomic sequences) encoding zeatin O-glucosyltransferase, a key enzyme in the regulation of zeatin activity in plants. The present invention also provides isolated plant nucleic acid molecules encoding O-xylosyltransferase, another key enzyme in the regulation of zeatin activity in plants.

10 In one embodiment, the zeatin O-glucosyltransferase nucleic acids disclosed are from the lima bean, *Phaseolus lunatus*. The open reading frame of these nucleic acid molecules encodes a polypeptide of 459 amino acids in length. This polypeptide is shown to have zeatin O-glucosyltransferase enzymatic activity i.e., it catalyzes the conversion of zeatin to O-glucosylzeatin. Accordingly, one aspect of the invention 15 comprises isolated nucleic acid molecules encoding zeatin O-glucosyltransferase. Another aspect of the invention is the purified zeatin O-glucosyltransferase enzyme.

15 In another embodiment, the zeatin O-xylosyltransferase nucleic acids disclosed are from *Phaseolus vulgaris*. The open reading frame of these nucleic acid molecules encodes a polypeptide of 454 amino acids in length. This polypeptide is shown to have zeatin O-xylosyltransferase enzymatic activity i.e., it catalyzes the conversion of zeatin to 20 O-xylosylzeatin. Accordingly, one aspect of the invention comprises isolated nucleic acid molecules encoding zeatin O-xylosyltransferase. Another aspect of the invention is the purified zeatin O-xylosyltransferase enzyme.

25 Also encompassed within the scope of this invention are transformation vectors which include at least a portion of the disclosed nucleic acid sequences. Such vectors may be transformed into plants to produce transgenic plants with modified zeatin O-glucosyltransferase activity and/or modified O-xylosyltransferase activity. Depending on the particular sequence incorporated into the vector, transformation with the zeatin O-glucosyltransferase cDNA and/or the O-xylosyltransferase cDNA, genes or derivatives 30 thereof may be used to modify agronomically important traits, including the activity of zeatin in seeds, grain yield and seed germination rates. While all crop plants may benefit from such modified activity, it is anticipated that the invention will be particularly valuable in maize, wheat and legumes.

35 Typically, vectors used to modify zeatin O-glucosyltransferase activity and/or O-xylosyltransferase activity include regulatory sequences that are operably linked to the zeatin O-glucosyltransferase cDNA and/or zeatin O-xylosyltransferase cDNA, gene(s), or derivatives thereof. For example, zeatin O-glucosyltransferase activity may be modified

in plants by introducing a transformation vector that includes a sense or antisense form of the disclosed cDNA operably linked to a high level constitutive promoter such as the 35S promoter of cauliflower mosaic virus. Transgenic plants transformed with such recombinant vectors and having modified zeatin O-glucosyltransferase activity and/or modified O-xylosyltransferase activity are part of the invention.

5 While the invention provides zeatin O-glucosyltransferase-encoding nucleic acids from *Phaseolus lunatus*, as well as zeatin O-xylosyltransferase-encoding nucleic acids from *Phaseolus vulgaris*, it additionally encompasses homologs, orthologs and derivatives of these sequences, as well as homologs, orthologs and variants of the zeatin O-
10 glucosyltransferase polypeptide sequence and the zeatin O-xylosyltransferase polypeptide sequence. Thus, in one aspect of the invention, nucleic acid molecules that comprise specified regions of these sequences are provided. Exemplary of such nucleic acid molecules are oligonucleotides that are useful as probes or primers to detect and amplify zeatin O-glucosyltransferase-encoding nucleic acids and zeatin O-xylosyltransferase-
15 encoding nucleic acids from other plant species. Such oligonucleotides are useful as hybridization probes or PCR primers, and typically comprise at least 15 consecutive bases of the disclosed sequences. In other embodiments, such oligonucleotides comprise longer regions of the disclosed sequences, such as at least 20, 25 or 30 consecutive nucleotides.

20 In another aspect, the invention provides compositions and methods for isolating nucleic acid sequences that have zeatin O-glucosyltransferase activity or zeatin O-xylosyltransferase activity from other plant species. Typically, such methods involve hybridizing probes or primers derived from the disclosed *P. lunatus* and *P. vulgaris* sequences to nucleic acids obtained or derived from such other plant species.

25 Homologous and orthologous sequences to the *P. lunatus* zeatin O-glucosyltransferase, or the *P. vulgaris* zeatin O-xylosyltransferase nucleic acid and amino acid sequences share key functional and structural characteristics with the disclosed *Phaseolus* sequences. Functionally, such sequences encode (or comprise) a polypeptide that either catalyzes the O-glucosylation of zeatin or the O-xylosylation of zeatin.

30 Structurally, such sequences share a specified structural relationship with the disclosed sequences. By way of example, in certain embodiments, homologous amino acid sequences have at least 70% sequence identity with the *P. lunatus* zeatin O-glucosyltransferase amino acid sequence or the *P. vulgaris* zeatin O-xylosyltransferase amino acid sequence. In other embodiments, homologous nucleic acid sequences
35 hybridize under stringent conditions to the disclosed *P. lunatus* zeatin O-glucosyltransferase nucleic acid sequences or the *P. vulgaris* O-xylosyltransferase nucleic acid sequences.

-5-

Another aspect of the invention relates to the purified zeatin O-glucosyltransferase enzyme, and the purified zeatin O-xylosyltransferase enzyme. Having provided nucleic acid molecules that encode these enzymes, the invention facilitates the expression of zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase in heterologous systems, including *E. coli*, yeast and baculovirus expression systems. Thus, the invention permits the large scale production of the enzymes for agricultural and other applications.

5 In another aspect of the invention the promoter sequence of the *P. lunatus* zeatin O-glucosyltransferase gene is disclosed. This promoter sequence confers seed-
10 specific expression, and may be used to express a variety of nucleic acids in a seed-specific manner.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the formation of O-glucosylzeatin from zeatin catalyzed by the
15 enzyme zeatin O-glucosyltransferase.

Fig. 2 is a graph showing the analysis of enzymatic reaction products by HPLC.
Fig. 2A: cytokinin standards. Fig. 2B: reaction products after incubating ¹⁴C-zeatin with recombinant fusion protein. Fig. 2C: reaction products treated with β -glucosidase.

For Fig. 2B: 200 μ l of supernatant obtained from cell culture was incubated with
20 approximately 1 nmol of labeled zeatin and UDPG for 2 hrs. For Fig. 2C: Approximately 0.4 nmole of reaction product obtained from reactions similar to Fig. 2B was incubated with β -glucosidase for 4 hrs to reconvert the product to zeatin.

Fig. 3 shows the formation of O-xylosylzeatin from zeatin catalyzed by the
25 enzyme zeatin O-xylosyltransferase.

25

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

Seq. I.D. No. 1 shows the nucleic acid sequence of the *Phaseolus lunatus* zeatin O-glucosyltransferase cDNA. The sequence includes the 5' untranslated region (base pairs 1-32) and the open reading frame (base pairs 33-1412).

35 Seq. I.D. No.2. shows the nucleic acid sequence of the *Phaseolus lunatus* zeatin O-glucosyltransferase gene. The sequence includes the TATA-like region (base pairs 29-34) and the open reading frame (base pairs 142-1521).

-6-

Seq. I.D. No. 3 shows the nucleic acid sequence of the *Phaseolus lunatus* zeatin O-glucosyltransferase open reading frame.
Seq. I.D. No. 4 shows the amino acid sequence of the *Phaseolus lunatus* zeatin O-glucosyltransferase polypeptide.

5 Seq. I.D. No. 5 shows the amino acid sequence of the 56 kDa. fusion protein. This fusion protein comprises 33 amino acids of the α -peptide encoded by the vector (amino acids 1-33) and the 5' untranslated region (amino acids 34-44) and the open reading frame (amino acids 45-503) of zeatin O-glucosyltransferase.

10 Seq. I.D. No. 6 shows the seed-specific promoter region of the *Phaseolus lunatus* zeatin O-glucosyltransferase gene.

Seq. I.D. Nos. 7-10 show primers that may be used to amplify various regions of the zeatin O-glucosyltransferase cDNA sequence.

Seq. I.D. No. 11 shows the nucleic acid sequence of the *Phaseolus vulgaris* zeatin O-xylosyltransferase open reading frame.

15 Seq. I.D. No. 12 shows the deduced amino acid sequence of the open reading frame of the *Phaseolus vulgaris* zeatin O-xylosyltransferase.

Seq. I.D. Nos. 13 and 14 show primers that may be used in conjunction with inverse PCR to identify sequences related to the zeatin O-glucosyltransferase sequence.

20 Seq. I.D. Nos. 15 and 16 show primers that may be used to amplify various regions of the zeatin O-xylosyltransferase cDNA sequence.

DETAILED DESCRIPTION OF THE INVENTION

25 I. Definitions and Abbreviations

A. Abbreviations

[¹⁴C]zeatin: *trans*-[8-¹⁴C]zeatin

Cis-Z: Cis-zeatin

Z: *trans*-zeatin

30 OGZ: O-glucosylzeatin

OXZ: O-xylosylzeatin

DHZ: dihydrozeatin

OXDHZ: O-xylosyldihydrozeatin

RZ: ribosylzeatin

35 OXRZ: O-xylosylribosylzeatin

UDPG: uridine diphosphate glucose

UDPX: uridine diphosphate xylose

ADPG: adenosine diphosphate glucose

TEA: triethylamine

ORF: open reading frame

MAb: monoclonal antibody

B. Definitions

5 Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V* published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew et al (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular 10 Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8). The nomenclature for DNA bases as set forth at 37 CFR § 1.822 and the standard three letter codes for amino acid residues 15 are used herein.

In order to facilitate review of the various embodiments of the invention, the 15 following definitions of terms is provided:

Zeatin O-glucosyltransferase: The defining functional characteristic of the zeatin O-glucosyltransferase enzyme is its ability to glucosylate cytokinins, such as zeatin. By way of example, the *Phaseolus* zeatin O-glucosyltransferase is capable of glucosylating zeatin to form O-glucosylzeatin, a conversion that is depicted in Fig. 1. 20 This activity can be measured using the assay described by Dixon et al. (1989), which is described in detail below. This invention provides a cDNA and a gene encoding the zeatin O-glucosyltransferase enzyme from *Phaseolus lunatus*. However the invention is not limited to this particular zeatin O-glucosyltransferase: other nucleotide sequences 25 which encode zeatin O-glucosyltransferase enzymes are also part of the invention, including variants on the disclosed *Phaseolus* cDNA and gene sequences and orthologous sequences from other plant species, the cloning of which is now enabled.

Such sequences share the essential functional characteristic of encoding an enzyme 30 that is capable of glucosylating cytokinins. Nucleic acid sequences that encode zeatin O-glucosyltransferases and the proteins encoded by such nucleic acids share not only this functional characteristic, but also a specified level of sequence similarity (or sequence identity), as addressed below. The concept of sequence identity can also be expressed in the ability of two sequences to hybridize to each other under stringent 35 conditions.

Zeatin O-xylosyltransferase: The defining functional characteristic of the zeatin O-xylosyltransferase enzyme is its ability to xylosylate cytokinins, such as zeatin. By way of example, the *Phaseolus* zeatin O-xylosyltransferase is capable of xylosylating zeatin to form O-xylosylzeatin, a conversion that is depicted in Fig. 3. This activity

can be measured using the assay described by Dixon et al. (1989), which is described in detail below. This invention provides a cDNA encoding the zeatin O-xylosyltransferase enzyme from *Phaseolus vulgaris*. However the invention is not limited to this particular zeatin O-xylosyltransferase: other nucleotide sequences which encode zeatin O-xylosyltransferase enzymes are also part of the invention, including variants on the disclosed *Phaseolus* cDNA and gene sequences and orthologous sequences from other plant species, the cloning of which is now enabled. Such sequences share the essential functional characteristic of encoding an enzyme that is capable of xylosylating cytokinins. Nucleic acid sequences that encode zeatin O-xylosyltransferases and the proteins encoded by such nucleic acids share not only this functional characteristic, but also a specified level of sequence similarity (or sequence identity), as addressed below. The concept of sequence identity can also be expressed in the ability of two sequences to hybridize to each other under stringent conditions.

15 **Sequence identity:** the similarity between two nucleic acid sequences, or two amino acid sequences is expressed in terms of sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are.

20 The calculation of percentage of sequence identity for amino acid sequences may take into account conservative amino acid substitutions. Conservative amino acid substitutions involve the replacement of one amino acid residue with another residue having similar chemical and biological properties (e.g., charge or hydrophobicity). Such substitutions typically do not change the functional properties of the protein, and should therefore be accounted for in the calculation of sequence identity by assigning 25 a value that is in between values assigned for identity (i.e., no change at that amino acid position) and non-conservative residue changes. Thus, conservative amino acid changes are scored as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. For example, if an identical amino acid is given a score of one and a non-conservative substitution is given a score of zero, a conservative 30 substitution might be given a score of 0.5. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

35 Methods of alignment of sequences for comparison are well-known in the art. Various programs and alignment algorithms are described in: Smith and Waterman (1981); Needleman and Wunsch (1970); Pearson and Lipman (1988); Higgins and Sharp (1988); Higgins and Sharp (1989); Corpet et al. (1988); Huang et al. (1992);

and Pearson et al. (1994). Altschul et al. (1994) presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) is available from several sources, including the National Center for Biological Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at <http://www.ncbi.nlm.nih.gov/BLAST/>. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

Homologs of the *Phaseolus* zeatin O-glucosyltransferase and zeatin O-xylosyltransferase proteins are characterized by possession of at least 70% sequence identity counted over the full length alignment with the disclosed *Phaseolus* amino acid using the NCBI Blast 2.0, gapped blastp set to default parameters. Such homologous peptides will more preferably possess at least 75%, more preferably at least 80% and still more preferably at least 90% or 95% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs will possess at least 75% and more preferably at least 85% and more preferably still at least 90% or 95% sequence identity over short windows of 10-20 amino acids. Methods for determining sequence identity over such short windows are described at http://www.ncbi.nlm.nih.gov/BLAST/blast_FAQs.html. Homologs having the sequence identities described above will, in some embodiments, also possess the ability to O-glucosylate zeatin or O-xylosylate zeatin. The present invention provides not only the peptide homologs described above, but also nucleic acid molecules that encode such homologs.

Homologs of the *Phaseolus* zeatin O-glucosyltransferase and zeatin O-xylosyltransferase cDNA as well as homologs of the zeatin O-glucosyltransferase gene are similarly characterized by possession of at least 60% sequence identity counted over the full length alignment with the disclosed *Phaseolus* cDNAs or gene sequences using the NCBI Blast 2.0, gapped blastn set to default parameters. Such homologous nucleic acids will more preferably possess at least 70%, more preferably at least 80% and still more preferably at least 90% or 95% sequence identity determined by this method. When less than the entire sequence is being compared for sequence identity, homologs will possess at least 85% and more preferably at least 90% and more preferably still at least 95% sequence identity over 30 nucleotide windows. Homologs having the sequence identities described above will, in some embodiments, also encode a polypeptide having ability to O-glucosylate or O-xylosylate zeatin. However, homologs as defined above are useful for modifying zeatin O-glucosyltransferase

-10-

activity and/or zeatin O-xylosyltransferase activity in transgenic plants (for example, as used in antisense constructs) even when they do not encode a functional peptide.

Another indication that two nucleic acid molecules are substantially homologous is that the two molecules hybridize to each other under stringent conditions when one molecule is used as a hybridization probe, and the other is present in a biological sample, e.g., genomic material from a cell. Specific hybridization means that the molecules hybridize substantially only to each other and not to other molecules that may be present in the genomic material. Stringent conditions are sequence dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Conditions for nucleic acid hybridization and calculation of stringencies can be found in Sambrook et al. (1989) and Tijssen (1993). Hybridization conditions and stringencies are further discussed below.

Nucleic acid sequences that do not show a high degree of identity may nevertheless encode similar amino acid sequences, due to the degeneracy of the genetic code. It is understood that changes in nucleic acid sequence can be made using this degeneracy to produce multiple nucleic acid sequence that all encode substantially the same protein.

Probes and primers: Nucleic acid probes and primers may readily be prepared based on the nucleic acids provided by this invention. A probe comprises an isolated nucleic acid attached to a detectable label or reporter molecule. Typical labels include radioactive isotopes, ligands, chemiluminescent agents, and enzymes. Methods for labeling and guidance in the choice of labels appropriate for various purposes are discussed, e.g., in Sambrook et al. (1989) and Ausubel et al. (1987).

Primers are short nucleic acids, preferably DNA oligonucleotides 15 nucleotides or more in length. Primers may be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, and then extended along the target DNA strand by a DNA polymerase enzyme.

Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Methods for preparing and using probes and primers are described, for example, in Sambrook et al. (1989), Ausubel et al. (1987), and Innis et al., (1990). PCR primer pairs can be derived from a known sequence, for example, by using

computer programs intended for that purpose such as Primer (Version 0.5, ©1991, Whitehead Institute for Biomedical Research, Cambridge, MA). One of skill in the art will appreciate that the specificity of a particular probe or primer increases with its length. Thus, for example, a primer comprising 20 consecutive nucleotides of the 5 *Phaseolus* zeatin O-glucosyltransferase cDNA or gene will anneal to a target sequence (e.g., a corresponding zeatin O-glucosyltransferase from *Zea mays*) with a higher specificity than a corresponding primer of only 15 nucleotides. Thus, in order to obtain greater specificity, probes and primers may be selected that comprise 20, 25, 10 30, 35, 40, 50 or more consecutive nucleotides of the *Phaseolus* zeatin O-glucosyltransferase cDNA or gene sequences.

Transformed: A transformed cell is a cell into which has been introduced a nucleic acid molecule by molecular biology techniques. As used herein, the term transformation encompasses all techniques by which a nucleic acid molecule might be introduced into such a cell, including transfection with viral vectors, transformation 15 with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration.

Isolated: An "isolated" biological component (such as a nucleic acid or protein) has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other 20 chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

25 **Purified:** The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified zeatin O-glucosyltransferase preparation is one in which the zeatin O-glucosyltransferase is more enriched than the protein is in its natural environment within a cell. Preferably, a preparation of zeatin O-glucosyltransferase is purified such that the zeatin O-glucosyltransferase represents at 30 least 50% of the total protein content of the preparation.

35 **Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

5 **Recombinant:** A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques.

10 **Ortholog:** two nucleotide or amino acid sequences are orthologs of each other if they share a common ancestral sequence and diverged when a species carrying that ancestral sequence split into two species. Orthologous sequences are also homologous sequences.

15 **Transgenic plant:** as used herein, this term refers to a plant that contains recombinant genetic material not normally found in plants of this type and which has been introduced into the plant in question (or into progenitors of the plant) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant which contain the introduced DNA (whether produced sexually or asexually).

20 **II. Isolation of cDNA and Genomic Sequences encoding Zeatin O-glucosyltransferase and Zeatin O-xylosyltransferase from *Phaseolus***

25 **A. Overview of Experimental Procedures**

30 The experimental procedure used to isolate the zeatin O-glucosyltransferase nucleic acid sequence involved creating a cDNA expression library and probing the library using a monoclonal antibody to zeatin O-glucosyltransferase. The clones that reacted with the antibody were transformed into a bacterial expression system that allowed a larger amount of the protein to be isolated. The isolated protein was then tested for zeatin O-glucosyltransferase activity.

35 Clones that contained the appropriate enzymatic activity were subjected to partial sequencing. This process allowed primers to be made that were specific to the newly isolated sequence. Subsequent use of inverse PCR allowed the entire gene to be isolated from genomic DNA. The PCR product from the genomic DNA was then cloned into a pGem-T vector (Promega) for complete sequencing.

40 The O-xylosyltransferase cDNA was identified through the use of inverse PCR followed by PCR. The inverse PCR primers were designed using the knowledge of the zeatin O-glucosyltransferase gene sequence. The sequence derived from the inverse PCR reaction then allowed specific PCR primers to be made. Finally, the PCR product was cloned into a pGem-T vector for complete sequencing.

45 Expression of the zeatin O-xylosyltransferase gene was achieved by subcloning the sequence into an inducible expression vector. The expressed protein was then

isolated and tested for enzymatic activity.

B. Methods

Construction of cDNA expression library and screening with MAbs to zeatin O-

5 **glucosyltransferase.** Total RNA was isolated from immature seeds of *P. lunatus* using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocols with modifications for samples with a high content of polysaccharides. mRNA was further enriched with the Promega PolyATract mRNA isolation system according to manufacturer's instructions (Promega, Madison, Wisc.).

10 An expression library was constructed using the SuperScript_{TM} Lambda System for cDNA synthesis and lambda ZipLox cloning (BRL Life Technologies, Inc.). First strand synthesis was primed with a *NotI*-oligo-d(T) primer-adapter. Following second strand synthesis *SaI* adapters were ligated to the cDNA, which was subsequently digested with *NotI*. This allowed for directional cloning of cDNA into the lambda ZipLox expression

15 vector (Gibco BRL, Gaithersburg, MD). The constructed lambda cDNA library was packaged following the manufacturer's instructions (BRL lambda packaging system). The lambda ZipLox cDNA library was plated with *E. coli* Y1090(ZL) on top agar and incubated at 37°C for 3-4 hours. The plates were then overlaid with nitrocellulose filters which had been presoaked with 10 mM IPTG and dried, and incubated at 37°C

20 overnight. Filters were marked for orientation, removed from the plates, and washed briefly in TBS. The membranes were blocked in TBS containing 2% nonfat dry milk for 1-2 hours at room temperature with constant agitation. Filters were then incubated with blocking solution containing primary antibody for 2 hours at 30°C. Filters were washed three times (5 minutes each) in blocking solution. Secondary antibody (Rabbit anti-

25 mouse conjugated to Alkaline Phosphatase, Jackson Laboratories) in blocking solution was applied to the filters for 2 hours at 30°C. Filters were washed and the positive clones identified with an Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA). Immunopositive plaques were removed, replated, and rescreened until pure plaques were obtained.

30 **Subcloning and sequencing of the zeatin O-glucosyltransferase cDNA.** The phage containing the lambda ZipLox vector harboring the insert was used to infect *E. coli* cell line DH10B(ZIP), thus allowing *in vivo* excision of the cDNA clone from the pZL1 plasmid. pZL1 clones were purified using Qiagen Midi Columns (Qiagen, Chatsworth, CA). Clones were sequenced using an Applied Biosystems 370A DNA sequence analyzer. Primers synthesized on an Applied Biosystems DNA Synthesizer were used for sequencing the inner portions of the clones.

Isolation of recombinant zeatin O-glucosyltransferase proteins. To obtain

recombinant proteins, purified plasmids were transformed into Epicurian Coli BL21(DE3) pLysS Competent cells (Stratagene, LaJolla, CA) following the recommended protocol. Colonies were selected on AMP-LB plates. An individual colony was grown overnight in SOC-AMP media and 0.5 ml of the culture was used to inoculate 50 mls of SOC-AMP media. Induction was achieved with IPTG (0.5 ml of 0.5 M) after cells were allowed to grow for 3-4 hours (OD at 595 nm of 0.8-1.0). After 4 hours cells were collected and frozen at -80°C overnight. Cells were lysed the next day by thawing and resuspending in 0.5 ml of 0.2 M Tris pH 7.5 containing 1 mg/ml of lysozyme. DNAase (1 µg/10µl sample) was added after a 10-min. cycle of freeze-thaw at -80°C. Soluble proteins were collected after centrifugation. Recombinant proteins were further purified on a DEAE anion exchange column using the Bio-Rad Biologic liquid chromatography system.

Isolation of genomic sequence of zeatin O-glucosyltransferase from *P. lunatus*.

Isolation of the genomic sequence was based on the principles of PCR, and both standard and inverse PCR (Ochman et al. 1989) were used. DNA was isolated from *P. lunatus* cv. Kingston using a modified CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle 1990). PCR and inverse PCR was performed with primers homologous to the 5' and 3' regions of the cDNA clone. To obtain the genomic sequence upstream and downstream of the cDNA using inverse PCR, the genomic DNA was digested with *Hind*III. After digestion, the restriction enzyme was inactivated by heating the sample to 75°C for 10 minutes. After dilution, T₄ DNA ligase (Promega) was added to allow intramolecular ligation (circularization). The reaction was performed at 15°C for 24 hours. The DNA was precipitated and PCR reaction performed with primers designed to amplify regions outside the cDNA.

To obtain genomic sequence inclusive of the expressed region (cDNA), standard PCR reactions were performed using pairs of primers based on the sequence of the cDNA. The products obtained from PCR and inverse PCR reactions were analyzed on a 1% Sea Plaque gel. Bands of interest were excised and DNA was purified with Qiaex II Gel Extraction Kit (Qiagen, Santa Clarita, CA). The products were ligated into a pGem-T vector (Promega) for sequencing.

Isolation of zeatin O-xylosyltransferase genomic sequence. The sequence derived from the cloning of the zeatin O-glucosyltransferase gene allowed primers to be designed such that homologous genes could be identified. One such homologous gene was zeatin O-xylosyltransferase. This gene was identified through the use of inverse PCR, as described above. Briefly, DNA from *P. vulgaris* was digested with *Hind*III restriction enzyme, re-ligated and used as template for inverse PCR with the following primers, taken from the *P. lunatus* zeatin O-glucosyltransferase cDNA sequence:

-15-

5'-CAT GGA GAT GGG TTC TTT CAT TGC AC-3' (Seq. I.D. No. 13)

5'-CAA CAA CTG AAG CAC TCA CCA ACG-3' (Seq. I.D. No. 14)

5 The sequence derived from the inverse PCR product was then compared to the
zeatin O-glucosyltransferase sequence and upon finding regions of non-homology,
specific primers were made to the inverse PCR product sequence. These primers were
as follows:

5'-CCA AAG TCG ACA ATG GCT TTG AAT GAT G-3' (Seq. I.D. No. 15)

10 5'-GCT ATG CGG CCG CCT AAA TGG TAT GAC-3' (Seq. I.D. No. 16)

The O-xylosyltransferase gene was then amplified using these primers in a PCR
reaction with *P. vulgaris* genomic DNA as a template. The products were then ligated
into a pGem-T vector (Promega) for sequencing.

15 **Isolation of recombinant zeatin O-xylosyltransferase proteins.** Zeatin O-
xylosyltransferase protein was purified using the same techniques as described above.
Briefly, the coding sequences were ligated into an IPTG inducible plasmid which was
transformed into Epicurian Coli BL21(DE3) pLysS Competent cells (Stratagene, LaJolla,
CA). IPTG was then used to induce protein. Cells were subsequently lysed and the
20 protein was separated out through the use of ion exchange chromatography.

Assay for cytokinin conversion activity and analysis of reaction products.
Enzyme activity (zeatin O-glucosyltransferase and zeatin O-xylosyltransferase) was
determined using the method of Dixon et al. (1989), which is incorporated herein by
reference. Briefly, ¹⁴C-labeled cytokinins (specific activity of 24 mCi/mmol), glycosyl
25 donor (3 mM of UDPG or UDPX), MgCl₂ (0.05M) and ATP (0.5 mM) in 1 mM Tris, pH
8.0 were incubated with a specified amount of the recombinant protein at 27 °C. One
mL of cold ethanol was added after one hour, and the mixture was placed at 4 °C for 15
minutes and then centrifuged at 27,000g for 20 minutes. The supernatant was
concentrated to 100 µL *in vacuo* (Speed Vac Concentrator, Savant) and analyzed by
30 HPLC using a reversed phase C₁₈ column.

For HPLC analysis (also performed as described by Dixon et al., 1989), a
Beckman model 110A dual pump HPLC system with a reversed-phase column
(Ultrasphere ODS C₁₈, 5 µM particle size, 4.6 X 250 mm; Altex) was used. The aqueous
buffer consisted of 0.2M acetic acid adjusted to pH 3.5 with TEA. Samples were eluted
35 with a linear gradient of methanol (5-50% over 90 minutes) in TEA buffer. The flow rate
was 1 mL/min. and 0.5 mL fractions were collected. A combination of a Beckman model
117 flow-through isotope detector and Isco UV monitor allowed the initial detection of

fractions of interest. Radioactivity in these fractions was determined in Ready-Gel scintillation fluid (Beckman) with a Beckman LS 7000 scintillation counter.

C. Results

5 **Isolation and authentication of zeatin O-glucosyltransferase cDNA clones.**

Approximately 10^6 plaques were screened with the MAb and nine immuno-positive lambda clones were selected. These were plaque purified and moved into cell line DH10B(ZIP) to allow circularization of the Zip-Lox plasmid containing the respective inserts. Epicurian Coli BL21(DE3) pLysS cells (Stratagene, LaJolla, Ca) transformed with 10 the purified plasmids were used to obtain the recombinant fusion proteins after IPTG induction. Supernatants were assayed for zeatin O-glucosyltransferase activity and for the presence of antigenic proteins by Western analysis. Recombinant protein from two clones, 21G and 27G, efficiently converted ^{14}C -zeatin to ^{14}C -O-glucosylzeatin (Fig. 2B) with UDPG as the glucosyl donor. The authenticity of the O-glucosyl product was 15 confirmed by treatment with β -glucosidase resulting in reconversion to zeatin (Fig. 2C). Western analyses confirmed the presence of antigenic protein of the expected size (56 kD) for the fusion protein (the polypeptide encoded by the ORF plus the 5' untranslated region and the α -peptide from the cloning vector). The native zeatin O-glucosyltransferase of *P. lunatus* also uses UDPX as the donor to form O-xylosylzeatin 20 but at a much lower affinity (Dixon et al. 1989).

To further test the authenticity of the recombinant protein encoded by clone 21G, UDPX was used as the glycosyl donor under the same reaction condition. As expected, O-xylosylzeatin was formed (see Table 1) but the amount was much lower. These results indicate that the selected cDNAs encode the correct sequence for zeatin O-glucosyltransferase. A trace of O-glucosyldihydrozeatin was formed when labeled 25 dihydrozeatin was incubated with the DEAE-column purified recombinant protein.

Table 1

Substrate Specificity of Recombinant Zeatin O-glucosyltransferase Encoded by a Clone from the cDNA Library

<u>Substrates</u>	<u>Product</u>	<u>Substrate Left</u>
	CPM (%)	CPM (%)
A. Z + UDPG	<u>OGZ</u> 39715 (100%)	<u>Zeatin</u> 0 (0%)
Z + UDPX	<u>OXZ</u> 4956 (18%)	<u>Zeatin</u> 23078 (82%)
B. Z + UDPG	<u>OGZ</u> 3731 (14%)	<u>Zeatin</u> 23115 (86%)
DHZ + UDPG	<u>O-G-DH-Zeatin</u> 329 (1%)	<u>DHZ</u> 24178 (99%)

5 A. 200 μ l of supernatant from lysed cells containing the recombinant protein was incubated with approximately 1 nmole of 14 C-labeled zeatin for 2 hr. and the reaction products separated by HPLC. UDPG as the glycosyl donor resulted in complete conversion of zeatin to O-glucosylzeatin while UDPX serving as the glycosyl donor resulted in much lower formation of O-xylosylzeatin.

10 B. DEAE-purified proteins (4 μ g) were incubated with approximately 0.5 nmole of 14 C-cytokinin

15 Isolation and authentication of zeatin O-xylosyltransferase cDNA clones. To assess the enzyme activity of the protein encoded by zeatin O-xylosyltransferase, the ORF of the gene was excised from the pGem-T vector (Promega) using the restriction enzymes *Not*1 and *Sal*1. The resulting fragment was then ligated into the *Not*1/*Sal*1 site of the pZipLox plasmid. This ligation resulted in an in-frame construct producing a fusion protein (with α -peptide) of 54 kD which is also antigenic to the monoclonal antibody (Martin et al. 1990). This allowed for the recombinant protein to be easily purified.

20 The product has high enzyme activity, converting radio-labeled zeatin to O-xylosylzeatin in the presence of UDPX (Table 2). The substrate specificity and the properties of the recombinant protein are identical to those of the native enzyme (Table 2). The result indicated that the clone encoded the zeatin O-xylosyltransferase protein.

Table 2
Substrate Specificity of Recombinant Protein Encoded by the ORF of Zeatin O-xylosyltransferase

<u>Substrates</u>	<u>Product</u>	<u>Substrate Left</u>	
		CPM (%)	CPM (%)
Z + UDPX	<u>OXZ</u>		<u>Z</u>
	35838 (82%)	7768 (18%)	
Z + UDPG	<u>OGZ</u>		<u>Z</u>
	ND.	41679 (100%)	
DHZ + UDPX	<u>OXDHZ</u>		<u>DHZ</u>
	33237 (78%)	9244 (22%)	
cis-Z + UDPX	<u>OX-cis-Z</u>		<u>cis-Z</u>
	ND.	29460 (100%)	
RZ + UDPX	<u>OXRZ</u>		<u>RZ</u>
	ND.	39238 (100%)	

5 Assay conditions: 125 μ l of protein from culture supernatant was incubated with radio-labeled cytokinins and UDPX or UDPG as indicated for 4 hrs. Reaction products were separated by HPLC according to Dixon et al. 1989.

10 **Distinguishing Zeatin O-glucosyltransferase from Zeatin O-xylosyltransferase.** A comparison between the results provided in Table 1, and the results provided in Table 2, confirms that the newly isolated recombinant zeatin O-glucosyltransferase protein and newly isolated recombinant zeatin O-xylosyltransferase protein are functionally 15 distinguishable. As shown in Table 2, the isolated zeatin O-xylosyltransferase protein was specific for the UPDX substrate. In fact there was no detectable conversion of zeatin when UDPG was used as the sugar donor (Table 2). However, when zeatin O-glucosyltransferase was used under the same reaction conditions 100% of the zeatin was utilized to form OGZ (Table 1).

20 **Sequence of Zeatin O-glucosyltransferase cDNA.** The sequence of zeatin O-glucosyltransferase cDNA obtained from *P. lunatus* is presented in Seq. I.D. No. 1. The ORF (open reading frame) is shown separately in Seq. I.D. No. 3. The cDNA is 1.5 kb long and contains *Ncol* and *Hind*III restriction sites. BLAST search of GENBANK and EMBL located no sequences showing significant homology. The amino acid sequence of the zeatin O-glucosyltransferase polypeptide is shown in Seq. I.D. No. 4.

25 **Genomic sequence of zeatin O-glucosyltransferase of *P. lunatus*.** The genomic sequence of zeatin O-glucosyltransferase is presented in Seq. I.D. No. 2. The sequence contains no introns. The TATA-like region is located around base-pairs 29-34.

Genomic sequence of zeatin O-xylosyltransferase of *P. vulgaris*. A genomic clone of 1390 bp was obtained from *Phaseolus vulgaris* cv. Great Northern. The

sequence of the product was determined. The gene contains an ORF of 1365 bp (Seq. I.D. No. 11) encoding a polypeptide of 454 amino acids (Seq. I.D. No. 12) with the deduced mass of 51.0 kD.

Comparison of the zeatin O-glucosyltransferase and the zeatin O-

5 **xylosyltransferase genes.** Like the zeatin O- glucosyltransferase gene, the zeatin O- xylosyltransferase gene does not contain introns. Additionally, the nucleotide sequences of the two genes are very similar with the percent identity at 93.0% over 1,380 base pairs of the zeatin O-glucosyltransferase ORF sequence (Seq. I.D. 3). The deduced amino acid sequences have a percent similarity of 90.4% and a percent
10 identity of 86.6% over 449 amino acids. These comparisons were determined using the program BESTFIT, with a gap setting of 12 which is available from Genetic Computing Group, Madison, WI.

EXAMPLES

15 The following examples are illustrative of various embodiments of the present invention.

20 **Example One: Preferred method for producing Zeatin O-glucosyltransferase and Zeatin O-xylosyltransferase nucleic acids**

With the provision herein of the zeatin O-glucosyltransferase cDNA sequence, the zeatin O-glucosyltransferase gene sequence and the zeatin O-xylosyltransferase ORF sequence (the disclosed *Phaseolus* sequences), the polymerase chain reaction (PCR) may now be utilized in a preferred method for producing nucleic acid sequences
25 encoding zeatin O-glucosyltransferase or zeatin O-xylosyltransferase. PCR amplification of the disclosed *Phaseolus* sequences may be accomplished either by direct PCR from a plant cDNA library or by Reverse-Transcription PCR (RT-PCR) using RNA extracted from plant cells as a template. Methods and conditions for both direct PCR and RT-PCR are known in the art and are described in Innis et al. (1990). Suitable plant
30 cDNA libraries for direct PCR include the *Arabidopsis* cDNA library described by Newman et al. (1994) and *Phaseolus* cDNA libraries constructed as described above.

The selection of PCR primers will be made according to the portions of the cDNA or gene that are to be amplified. Primers may be chosen to amplify small segments of the cDNA, the open reading frame, the entire cDNA molecule or the entire
35 gene sequence. Variations in amplification conditions may be required to accommodate primers of differing lengths; such considerations are well known in the art and are discussed in Innis et al. (1990), Sambrook et al. (1989), and Ausubel et al. (1992). By way of example only, the entire zeatin O-glucosyltransferase cDNA

molecule as shown in Seq. I.D. No. 1 may be amplified using the following combination of primers:

5'-GAAGCATAGCATCTTGCTACC-3' (Seq. I.D. No. 7)

5'-CAACAGAAGCTTATTATTGG-3' (Seq. I.D. No. 8)

5

The open reading frame portion of the cDNA may be amplified using the following primer pair:

5'-ATGGCTTGAAATGACAAAAGC-3' (Seq. I.D. No. 9)

10 5'-CTATTTAGAGATGTGTGCAATG-3' (Seq. I.D. No. 10)

These primers are illustrative only; it will be appreciated by one skilled in the art that many different primers may be derived from the provided cDNA and gene sequences in order to amplify particular regions of these molecules. Resequencing of PCR products obtained by these amplification procedures is recommended; this will facilitate confirmation of the amplified sequence and will also provide information on natural variation on this sequence in different ecotypes and plant populations.

Oligonucleotides that are derived from the disclosed *Phaseolus* sequences are encompassed within the scope of the present invention. Preferably, such oligonucleotide primers will comprise a sequence of at least 15-20 consecutive nucleotides of the disclosed *Phaseolus* sequences. To enhance amplification specificity, oligonucleotide primers comprising at least 25, 30, 35, 40, 45 or 50 consecutive nucleotides of these sequences may also be used.

25 **Example Two: Isolation of homologous gene sequence from other plant species**

With the provision herein of the disclosed *Phaseolus* sequences, the cloning of orthologous cDNAs and genes from other plant species by standard methodologies is now enabled. Thus, the present invention includes methods of isolating both cDNA and genomic sequences encoding zeatin O-glucosyltransferase and zeatin O-xylosyltransferase. Both conventional hybridization and PCR amplification procedures may be utilized to clone such sequences. Common to both of these techniques is the hybridization of probes or primers derived from the disclosed *Phaseolus* sequences to a target nucleotide preparation, which may be, in the case of conventional hybridization approaches, a cDNA or genomic library or, in the case of PCR amplification, a cDNA or genomic library, or an mRNA preparation.

30 35
35 Direct PCR amplification may be performed on cDNA or genomic libraries prepared from the plant species in question, or RT-PCR may be performed using mRNA

extracted from the plant cells using standard methods. PCR primers will comprise at least 15 consecutive nucleotides of the disclosed *Phaseolus* sequences. One of skill in the art will appreciate that sequence differences between the disclosed *Phaseolus* sequences and the target nucleic acid to be amplified may result in lower amplification 5 efficiencies. To compensate for this, longer PCR primers or lower annealing temperatures may be used during the amplification cycle. Where lower annealing temperatures are used, sequential rounds of amplification using nested primer pairs may be necessary to enhance specificity.

For conventional hybridization techniques (described further in Example four 10 below) the hybridization probe is preferably conjugated with a detectable label such as a radioactive label, and the probe is preferably of at least 20 nucleotides in length. As is well known in the art, increasing the length of hybridization probes tends to give enhanced specificity. The labeled probe derived from the *Phaseolus* cDNA or gene sequence may be hybridized to a plant cDNA or genomic library and the hybridization 15 signal detected using means known in the art. The hybridizing colony or plaque (depending on the type of library used) is then purified and the cloned sequence contained in that colony or plaque isolated and characterized.

Homologs of the *Phaseolus* zeatin O-glucosyltransferase and *Phaseolus* zeatin O-xylosyltransferase may alternatively be obtained by immunoscreening of an expression 20 library. With the provision herein of the disclosed *Phaseolus* nucleic acid sequences, the enzymes may be expressed and purified in a heterologous expression system (e.g., *E. coli*) and used to raise antibodies (monoclonal or polyclonal) specific for the zeatin O-glucosyltransferase protein or the zeatin O-xylosyltransferase protein. Antibodies may also be raised against synthetic peptides derived from the *Phaseolus* amino acid 25 sequences presented herein. Methods of raising antibodies are well known in the art and are described in Harlow and Lane (1988). Such antibodies can then be used to screen an expression cDNA library produced from the plant from which it is desired to clone the zeatin O-glucosyltransferase or zeatin O-xylosyltransferase gene ortholog, using the methods described above. The selected cDNAs can be confirmed by sequencing and 30 enzyme activity.

The disclosed *Phaseolus* sequences, and homologs of these sequences from other plants may be incorporated into transformation vectors and introduced into plants to modify zeatin O-glucosyltransferase activity and/or zeatin O-xylosyltransferase activity in such plants, as described in Example three below. It is anticipated that the 35 native zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase gene promoter may be particularly useful in the practice of the present invention in that it may be used to drive the expression of zeatin O-glucosyltransferase and/or zeatin O-

xylosyltransferase transgenes, such as antisense constructs. For example, by using the native zeatin O-glucosyltransferase gene promoter, expression of these transgenes may be regulated in coordination with the native zeatin O-glucosyltransferase gene (for example, in the same temporal or tissue-specific expression patterns).

5

Example three: Transgenic plants with modified zeatin O-glucosyltransferase and/or Zeatin O-xylosyltransferase expression

Once a gene (or cDNA) encoding a protein involved in the determination of a 10 particular plant characteristic has been isolated, standard techniques may be used to express the cDNA in transgenic plants in order to modify that particular plant characteristic. The basic approach is to clone the cDNA into a transformation vector, such that it is operably linked to control sequences (e.g., a promoter) that direct 15 expression of the cDNA in plant cells. The transformation vector is then introduced into plant cells by one of a number of techniques (e.g., electroporation) and progeny plants containing the introduced cDNA are selected. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part 20 of the transformation vector which integrates into the plant cell and which contains the introduced cDNA and associated sequences for controlling expression (the introduced "transgene") may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of an altered phenotype. Such a phenotype may result directly from the cDNA cloned into the transformation vector or may be manifested as enhanced resistance to a chemical agent (such as an antibiotic) as a result of the 25 inclusion of a dominant selectable marker gene incorporated into the transformation vector.

The choice of (a) control sequences and (b) how the cDNA (or selected portions of the cDNA) are arranged in the transformation vector relative to the control sequences determine, in part, how the plant characteristic affected by the introduced 30 cDNA is modified. For example, the control sequences may be tissue specific, such that the cDNA is only expressed in particular tissues of the plant (e.g., pollen, seed) and so the affected characteristic will be modified only in those tissues. The cDNA sequence may be arranged relative to the control sequence such that the cDNA transcript is expressed normally, or in an antisense orientation. Expression of an 35 antisense RNA corresponding to the cloned cDNA will result in a reduction of the targeted gene product (the targeted gene product being the protein encoded by the plant gene from which the introduced cDNA was derived). Over-expression of the introduced cDNA, resulting from a plus-sense orientation of the cDNA relative to the control sequences in the vector, may lead to an increase in the level of the gene

product, or may result in co-suppression (also termed "sense suppression") of that gene product.

Successful examples of the modification of plant characteristics by transformation with cloned cDNA sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the current knowledge in this field of technology, and which are herein incorporated by reference, include:

U.S. Patent No. 5,451,514 to Boudet (modification of lignin synthesis using antisense RNA and co-suppression);

U.S. Patent No. 5,443,974 to Hitz (modification of saturated and unsaturated fatty acid levels using antisense RNA and co-suppression);

U.S. Patent No. 5,530,192 to Murase (modification of amino acid and fatty acid composition using antisense RNA);

U.S. Patent No. 5,455,167 to Voelker (modification of medium chain fatty acids);

U.S. Patent No. 5,231,020 to Jorgensen (modification of flavenoids using co-suppression);

U.S. Patent No. 5,583,021 to Dougherty (modification of virus resistance by expression of plus-sense untranslatable RNA);

WO 96/13582 (modification of seed VLCFA composition using over expression, co-suppression and antisense RNA in conjunction with the *Arabidopsis FAE1* gene); and

WO 95/15387 (modification of seed VLCFA composition using over expression of jojoba wax synthesis gene).

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to over-express the introduced cDNA or to express antisense RNA corresponding to the cDNA. In light of the foregoing and the disclosed *Phaseolus* sequences, it is thus apparent that one of skill in the art will be able to introduce these nucleic acids, or homologous or derivative forms of these molecules (e.g., antisense forms), into plants in order to produce plants having modified zeatin O-glucosyltransferase activity and/or modified zeatin O-xylosyltransferase activity. Modification of the activity of zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase in plants will permit controlled modification of not only zeatin function, but also other cytokinins and, as a consequence of the interdependent regulation of plant hormones, other hormones. The result can be altered plant development with agricultural and economic consequences.

a. **Plant Types**

Zeatins are found in all plant types, and thus DNA molecules according to the

present invention (e.g., the zeatin O-glucosyltransferase cDNA, the zeatin O-xylosyltransferase ORF, and the glucosyltransferase gene, homologs of these sequences and derivatives such as antisense forms) may be introduced into any plant type in order to modify the zeatin O-glucosyltransferase activity or the zeatin O-

5 xylosyltransferase activity in the plant. Thus, the sequences of the present invention may be used to modify zeatin O-glucosyltransferase activity and/or zeatin O-xylosyltransferase activity in any higher plant, including monocotyledonous, dicotyledonous and gymnosperm plants, including, but not limited to maize, wheat, rice, barley, soybean, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, 10 brassica, pine trees such as the loblolly pine and Douglas fir, cotton, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, potato, carrot, radish, pea, lentils, cabbage, broccoli, brussel sprouts, peppers; tree fruits such as apples, pears, peaches, apricots; flowers such as carnations and roses.

15 **b. Vector Construction, Choice of Promoters**

A number of recombinant vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described including those described in Pouwels et al., (1987), Weissbach and Weissbach, (1989), and Gelvin et al., (1990). Typically, plant transformation vectors include one or more cloned plant 20 genes (or cDNAs) under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant transformation vectors typically also contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally-or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA 25 processing signal, a transcription termination site, and/or a polyadenylation signal.

Examples of constitutive plant promoters which may be useful for expressing the cDNA include: the cauliflower mosaic virus (CaMV) 35S promoter, which confers constitutive, high-level expression in most plant tissues (see, e.g., Odel et al., 1985, Dekeyser et al., 1990, Terada and Shimamoto, 1990; Benfey and Chua, 1990); the 30 nopaline synthase promoter (An et al., 1988); and the octopine synthase promoter (Fromm et al., 1989).

A variety of plant gene promoters that are regulated in response to environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of the cDNA in plant cells, including promoters regulated by: (a) heat (Callis et al., 1988; Ainley, et al. 1993; Gilmartin et al. 1992); (b) light (e.g., the pea rbcS-3A promoter, Kuhlemeier et al., 1989, and the maize rbcS promoter, Schaffner and Sheen, 1991); (c) hormones, such as abscisic acid (Marcotte et al., 1989); (d) wounding (e.g., *wun1*, Siebertz et al., 1989); and (e) chemicals such as methyl jasminate or salicylic

acid (see also Gatz et al., 1997) can also be used to regulate gene expression.

Alternatively, tissue specific (root, leaf, flower, and seed for example) promoters (Carpenter et al. 1992, Denis et al. 1993, Opperman et al. 1993, Stockhause et al. 1997; Roshal et al., 1987; Scherthaner et al., 1988; and Bustos et al., 1989) can be fused to the coding sequence to obtain particular expression in respective organs. In addition, the timing of the expression can be controlled by using promoters such as those acting at senescence (Gan and Amasino 1995) or late seed development (Odell et al. 1994).

The promoter region of the zeatin O-glucosyltransferase gene sequence disclosed herein (Shown in Seq. I.D. No. 6) confers seed-specific expression in *P. lunatus*.

Accordingly, the native promoter may be used to obtain seed specific expression of the introduced transgene.

Plant transformation vectors may also include RNA processing signals, for example, introns, which may be positioned upstream or downstream of the ORF sequence in the transgene. In addition, the expression vectors may also include additional regulatory sequences from the 3'-untranslated region of plant genes, e.g., a 3' terminator region to increase mRNA stability of the mRNA, such as the PI-II terminator region of potato or the octopine or nopaline synthase 3' terminator regions.

Finally, as noted above, plant transformation vectors may also include dominant selectable marker genes to allow for the ready selection of transformants. Such genes include those encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (e.g., phosphinothricin acetyltransferase).

25 **c. Arrangement of zeatin O-glucosyltransferase and/or O-xylosyltransferase sequence in vector**

The particular arrangement of the zeatin O-glucosyltransferase sequence and/or zeatin O-xylosyltransferase sequence in the transformation vector will be selected according to the type of expression of the sequence that is desired.

Where enhanced zeatin O-glucosyltransferase activity and/or enhanced zeatin O-xylosyltransferase activity is desired in the plant, the zeatin O-glucosyltransferase ORF and/or the zeatin O-xylosyltransferase ORF may be operably linked to a constitutive high-level promoter such as the CaMV 35S promoter. As noted below, modification of zeatin O-glucosyltransferase synthesis and/or zeatin O-xylosyltransferase synthesis may also be achieved by introducing into a plant a transformation vector containing a variant form of the disclosed *Phaseolus* sequences. For example, a form can vary from the exact nucleotide sequence of the zeatin O-glucosyltransferase ORF, but still encode a protein that retains the functional characteristic of the zeatin O-glucosyltransferase

protein, i.e., O-glucosylation of cytokinins, such as zeatin.

In contrast, a reduction of zeatin O-glucosyltransferase activity and/or zeatin O-xylosyltransferase activity in the transgenic plant may be obtained in a number of different ways. For example, a reduction in protein product can be achieved through the use of antisense sequences, ribozymes, co-suppression, untranslatable RNAs, and/or dominant negative mutants.

For antisense suppression, the disclosed *Phaseolus* sequences are arranged in reverse orientation relative to the promoter sequence in the transformation vector.

The introduced sequence need not be the full length of the disclosed *Phaseolus*

10 sequences, and need not be exactly homologous to the endogenous zeatin O-glucosyltransferase or the endogenous zeatin O-xylosyltransferase found in the plant type to be transformed. Generally, however, where the introduced sequence is of shorter length, a higher degree of homology to the endogenous zeatin O-glucosyltransferase sequence and/or the endogenous zeatin O-xylosyltransferase

15 sequence will be needed for effective antisense suppression. Preferably, the introduced antisense sequence in the vector will be at least 30 nucleotides in length, and improved antisense suppression will typically be observed as the length of the antisense sequence increases. Preferably, the length of the antisense sequence in the vector will be greater than 100 nucleotides. Transcription of an antisense construct as described results in the production of RNA molecules that are the reverse complement of mRNA molecules transcribed from the endogenous zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase gene(s) in the plant cell. Although the exact mechanism by which antisense RNA molecules interfere with gene expression has not been elucidated, it is believed that antisense RNA molecules bind to the endogenous 20 mRNA molecules and thereby inhibit translation of the endogenous mRNA.

Suppression of endogenous zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase expression can also be achieved using ribozymes. Ribozymes are synthetic RNA molecules that possess highly specific endoribonuclease activity. The production and use of ribozymes are disclosed in U.S. Patent No. 4,987,071 to Cech and U.S. Patent No. 5,543,508 to Haselhoff. The inclusion of ribozyme sequences 30 within antisense RNAs may be used to confer RNA cleaving activity on the antisense RNA, such that endogenous mRNA molecules that bind to the antisense RNA are cleaved, which in turn leads to an enhanced antisense inhibition of endogenous gene expression.

35 Constructs in which the disclosed *Phaseolus* sequences (or variants thereof) are over-expressed may also be used to obtain co-suppression of the endogenous zeatin O-glucosyltransferase and/or the endogenous zeatin O-xylosyltransferase in the

manner described in U.S. Patent No. 5,231,021 to Jorgensen. Such co-suppression (also termed sense suppression) does not require that the entire disclosed *Phaseolus* sequence be introduced into the plant cells, nor does it require that the introduced sequence be exactly identical to the endogenous zeatin O-glucosyltransferase sequence and/or the endogenous zeatin O-xylosyltransferase sequence. However, as with antisense suppression, the suppressive efficiency will be enhanced as (1) the introduced sequence is lengthened and (2) the sequence similarity between the introduced sequence and the endogenous zeatin O-glucosyltransferase gene and/or zeatin O-xylosyltransferase gene is increased.

10 Constructs expressing an untranslatable form of the zeatin O-glucosyltransferase mRNA and/or an untranslatable form of zeatin O-xylosyltransferase mRNA may also be used to suppress the expression of endogenous zeatin O-glucosyltransferase and/or endogenous zeatin O-xylosyltransferase. Methods for producing such constructs are described in U.S. Patent No. 5,583,021 to Dougherty et al. Preferably, such constructs are made by introducing a premature stop codon into the zeatin O-glucosyltransferase ORF and/or the zeatin O-xylosyltransferase ORF.

15 Finally, dominant negative mutant forms of the disclosed *Phaseolus* proteins may be used to block endogenous zeatin O-glucosyltransferase activity or the endogenous zeatin O-xylosyltransferase activity. Such mutants require the production of 20 mutated forms of the zeatin O-glucosyltransferase protein or the zeatin O-xylosyltransferase protein that bind to zeatin but do not catalyze the enzymatic step.

d. Transformation and Regeneration Techniques

Transformation and regeneration of both monocotyledonous and 25 dicotyledonous plant cells is now routine, and the selection of the most appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated 30 transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium tumefaciens* (AT) mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

35

e. Selection of Transformed Plants

Following transformation and regeneration of plants with the transformation vector, transformed plants are preferably selected using a dominant selectable marker

incorporated into the transformation vector. Typically, such a marker will confer antibiotic resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic.

5 After transformed plants are selected and grown to maturity, they can be assayed using the methods described herein to determine whether zeatin O-glucosyltransferase activity and/or zeatin O-xylosyltransferase activity has been altered as a result of the introduced transgene. In addition, antisense or sense suppression of the endogenous zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase gene(s) 10 may be detected by analyzing mRNA expression on Northern blots.

Example four: Production of sequence variants

As noted above, modification of zeatin O-glucosyltransferase activity and/or zeatin O-xylosyltransferase activity in plant cells can be achieved by transforming 15 plants with the disclosed *Phaseolus* sequences, antisense constructs based on the disclosed *Phaseolus* sequences, or other variants of the disclosed *Phaseolus* sequences. With the provision of the disclosed *Phaseolus* sequences herein, the creation of variants on these sequences by standard mutagenesis techniques is now enabled.

20 Variant DNA molecules include those created by standard DNA mutagenesis techniques, for example, M13 primer mutagenesis. Details of these techniques are provided in Sambrook et al. (1989), Ch. 15. By the use of such techniques, variants may be created which differ in minor ways from the disclosed *Phaseolus* sequences. DNA molecules and nucleotide sequences which are derivatives of those specifically 25 disclosed herein and which differ from those disclosed by the deletion, addition or substitution of nucleotides while still encoding a protein which possesses the functional characteristic of the zeatin O-glucosyltransferase protein, or the functional characteristic of the zeatin O-xylosyltransferase protein (i.e., the ability to convert zeatin to O-glucosylzeatin or O-xylosylzeatin) are comprehended by this invention. DNA 30 molecules and nucleotide sequences which are derived from the disclosed *Phaseolus* sequences include DNA sequences which hybridize under stringent conditions to the DNA sequences disclosed, or fragments thereof.

Hybridization conditions resulting in particular degrees of stringency will vary 35 depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing DNA used. Generally, the temperature of hybridization and the ionic strength (especially the Na⁺ concentration) of the hybridization buffer will determine the stringency of hybridization. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by

Sambrook et al. (1989), chapters 9 and 11, herein incorporated by reference. By way of illustration only, a hybridization experiment may be performed by hybridization of a DNA molecule (for example, a variant of the *Phaseolus* zeatin O-glucosyltransferase cDNA sequence) to a target DNA molecule (for example, the *Phaseolus* zeatin O-

5 glucosyltransferase cDNA sequence) which has been electrophoresed in an agarose gel and transferred to a nitrocellulose membrane by Southern blotting (Southern, 1975), a technique well known in the art and described in (Sambrook et al., 1989).

Hybridization with a target probe labeled with [³²P]-dCTP is generally carried out in a solution of high ionic strength such as 6xSSC at a temperature that is 20-25° C below 10 the melting temperature, T_m , described below. For such Southern hybridization experiments where the target DNA molecule on the Southern blot contains 10 ng of DNA or more, hybridization is typically carried out for 6-8 hours using 1-2 ng/ml radiolabeled probe (of specific activity equal to 10⁹ CPM/ μ g or greater). Following hybridization, the nitrocellulose filter is washed to remove background hybridization.

15 The washing conditions should be as stringent as possible to remove background hybridization but to retain a specific hybridization signal. The term T_m represents the temperature above which, under the prevailing ionic conditions, the radiolabeled probe molecule will not hybridize to its target DNA molecule. The T_m of such a hybrid molecule may be estimated from the following equation (Bolton and McCarthy, 1962):

20

$$T_m = 81.5 \text{ } ^\circ\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\% \text{ formamide}) - (600//)$$

Where / = the length of the hybrid in base pairs.

This equation is valid for concentrations of Na^+ in the range of 0.01 M to 0.4 M, and it 25 is less accurate for calculations of T_m in solutions of higher $[\text{Na}^+]$. The equation is also primarily valid for DNAs whose G + C content is in the range of 30% to 75%, and it applies to hybrids greater than 100 nucleotides in length (the behavior of oligonucleotide probes is described in detail in Ch. 11 of Sambrook et al., 1989).

30 Thus, by way of example, for a 150 base pair DNA probe derived from the first 150 base pairs of the open reading frame of the *Phaseolus* zeatin O-glucosyltransferase cDNA (with a hypothetical %GC = 45%), a calculation of hybridization conditions required to give particular stringencies may be made as follows:

For this example, it is assumed that the filter will be washed in 0.3 xSSC solution following hybridization, thereby $[\text{Na}^+] = 0.045\text{M}$; %GC = 45%;

35 Formamide concentration = 0; / = 150 base pairs; and $T_m = 81.5 \text{ } ^\circ\text{C} - 16(\log_{10}[\text{Na}^+]) + (0.41 \times 45) - (600/150)$ and so $T_m = 74.4 \text{ } ^\circ\text{C}$.

The T_m of double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology (Bonner et al., 1973). Therefore, for this given example, washing the filter in 0.3 xSSC at 59.4-64.4 °C will produce a stringency of hybridization equivalent to 90%. Alternatively, washing the hybridized filter in 0.3 xSSC at a temperature of 5 65.4-68.4 °C will yield a hybridization stringency of 94%. The above example is given entirely by way of theoretical illustration. One skilled in the art will appreciate that other hybridization techniques may be utilized and that variations in experimental conditions will necessitate alternative calculations for stringency.

10 DNA sequences from plants that encode a protein having zeatin O-glucosyltransferase activity and which hybridize under hybridization conditions of at least 75%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90% and most preferably at least 95% stringency are encompassed within the present invention.

15 The degeneracy of the genetic code further widens the scope of the present invention as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. For example, the second amino acid residue of the *Phaseolus* zeatin O-glucosyltransferase protein is alanine. This is encoded in the *Phaseolus* zeatin O-glucosyltransferase open reading frame by the nucleotide codon triplet GCT. Because of the degeneracy of the genetic 20 code, three other nucleotide codon triplets--GCA, GCC and GCG--also code for alanine. Thus, the nucleotide sequence of the *Phaseolus* zeatin O-glucosyltransferase ORF could be changed at this position to any of these three codons without affecting the amino acid composition of the encoded protein or the characteristics of the protein. Based upon the degeneracy of the genetic code, variant DNA molecules may be 25 derived from the cDNA and gene sequences disclosed herein using standard DNA mutagenesis techniques as described above, or by synthesis of DNA sequences. Thus, this invention also encompasses nucleic acid sequences which encode a zeatin O-glucosyltransferase protein but which vary from the disclosed nucleic acid sequences by virtue of the degeneracy of the genetic code.

30 One skilled in the art will recognize that DNA mutagenesis techniques may be used not only to produce variant DNA molecules, but will also facilitate the production of proteins which differ in certain structural aspects from the disclosed *Phaseolus* proteins, yet which proteins are clearly derivative of these proteins and which maintain the essential functional characteristics of zeatin O-glucosyltransferase or zeatin O-35 xylosyltransferase. Newly derived proteins may also be selected in order to obtain variations on the characteristics of the disclosed *Phaseolus* proteins, as will be more fully described below. Such derivatives include those with variations in amino acid

sequence including minor deletions, additions and substitutions.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be 5 conducted at the target codon or region and the expressed protein variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence as described above are well known.

Amino acid substitutions are typically of single residues; insertions usually will 10 be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions preferably are made in adjacent pairs, i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. Obviously, the mutations that are made in the DNA encoding the protein must not 15 place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure.

Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 3 when it is 20 desired to finely modulate the characteristics of the protein. Table 3 shows amino acids which may be substituted for an original amino acid in a protein and which are regarded as conservative substitutions.

Table 3
Listing of Conservative Amino Acid Substitutions

	Original Residue	Conservative Substitutions
5	Ala	ser
	Arg	lys
	Asn	gln; his
	Asp	glu
	Cys	ser
10	Gln	asn
	Glu	asp
	Gly	pro
	His	asn; gln
	Ile	leu, val
15	Leu	ile; val
	Lys	arg; gln; glu
	Met	leu; ile
	Phe	met; leu; tyr
	Ser	thr
20	Thr	ser
	Trp	tyr
	Tyr	trp; phe
	Val	ile; leu

25 Substantial changes in enzymatic function or other features are made by selecting substitutions that are less conservative than those in Table 3, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in protein properties will be those in which (a) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, e.g., glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having a side chain, e.g., glycine.

30 The effects of these amino acid substitutions or deletions or additions may be assessed for derivatives of the zeatin O-glucosyltransferase protein and/or the zeatin O-xylosyltransferase protein by analyzing the ability of the derivative proteins to catalyze the conversion of zeatin to O-glucosylzeatin or zeatin to O-xylosylzeatin. These assays 35 may conveniently be performed using the assay described above.

Example five: Production of recombinant zeatin O-glucosyltransferase or zeatin O-xylosyltransferase using heterologous expression systems

Many different expression systems are available for expressing cloned nucleic acid molecules. Examples of prokaryotic and eukaryotic expression systems that are routinely used in laboratories are described in Chapters 16-17 of Sambrook et al. (1989), which are herein incorporated by reference. Such systems may be used to express zeatin O-glucosyltransferase and/or zeatin O-xylosyltransferase at high levels to facilitate purification of the enzyme. The enzyme may be used for a variety of purposes. For example, it may be applied directly to plants to modulate zeatin function. Alternatively the purified enzyme produced by recombinant means may be used to synthesize O-glucosylzeatin, O-xylosylzeatin and other zeatin metabolites in vitro, particularly radio- or fluorescent-labeled forms of O-glucosylzeatin, O-xylosylzeatin and zeatin metabolites. These molecules may be used as tracers to determine the location in plant tissues and cells of zeatin and its metabolites. In addition, the recombinant form of the enzyme may be used to produce labeled forms of metabolites of other substrates (for example, isoprenylated proteins) on which it may act. The purified recombinant enzyme may also be used as an immunogen to raise enzyme-specific antibodies. Such antibodies are useful as both research reagents (such as in the study of zeatin regulation in plants) as well as diagnostically to determine expression levels of the enzyme in agricultural products, including seed.

By way of example only, high level expression of the zeatin O-glucosyltransferase-protein may be achieved by cloning and expressing the cDNA in yeast cells using the pYES2 yeast expression vector (Invitrogen, San Diego, CA). The recombinant zeatin O-glucosyltransferase may be supplied in the harvested yeast cells (for subsequent processing). Alternatively, a genetic construct may be produced to direct secretion of the recombinant zeatin O-glucosyltransferase from the yeast cells into the medium. This approach will facilitate the purification of the zeatin O-glucosyltransferase protein, if this is necessary. Secretion of the recombinant zeatin O-glucosyltransferase from the yeast cells may be achieved by placing a yeast signal sequence adjacent to the zeatin O-glucosyltransferase coding region. A number of yeast signal sequences have been characterized, including the signal sequence for yeast invertase. This sequence has been successfully used to direct the secretion of heterologous proteins from yeast cells, including such proteins as human interferon (Chang et al., 1986), human lactoferrin (Liang and Richardson, 1993) and prochymosin (Smith et al., 1985).

Alternatively, the enzyme may be expressed at high level in prokaryotic expression systems, such as *E. coli* as described above.

Example six: Use of the zeatin O-glucosyltransferase gene promoter sequence

The promoter of the *Phaseolus lunatus* zeatin O-glucosyltransferase gene confers 5 seed-specific expression. Accordingly, this promoter sequence, shown in Seq. I.D. No. 6, may be used to produce transgene constructs that are specifically expressed in seeds.

One of skill in the art will recognize that effective seed-specific expression may be achieved with less than the entire 141 base pair sequence shown in Seq. I.D. No. 6.

Thus, by way of example, seed-specific expression may be obtained by employing a 10 50 base pair or 100 base pair region of the disclosed promoter sequence. The determination of whether a particular sub-region of the disclosed sequence operates to confer effective seed-specific expression in a particular system (taking into account the plant species into 15 which the construct is being introduced, the level of expression required, etc.) will be performed using known methods, such as operably linking the promoter sub-region to a marker gene (e.g. GUS), introducing such constructs into plants and then determining the level of expression of the marker gene in seeds and other plant tissues.

The present invention therefore facilitates the production, by standard molecular biology techniques, of nucleic acid molecules comprising this promoter sequence 20 operably linked to a nucleic acid sequence, such as an open reading frame. Suitable open reading frames include open reading frames encoding any protein for which seed-specific expression is desired. Examples of genes that may suitably be expressed in a seed-specific manner under the control of the zeatin O-glucosyltransferase promoter include, but are not limited to:

(1) genes that enhance the nutritional quality of the seeds, for example, by 25 increasing the content of limiting amino acids, including lysine, methionine and cysteine.

This may be achieved by expressing proteins containing high levels of these amino acids in seeds. Examples include the high methionine storage proteins from brazil nut (Saalbach et al., 1996) and sunflower (Molvig et al., 1997).

(2) genes that increase gluten levels in wheat, so as to enhance the bread-making 30 quality of the wheat flour (Shewry et al., 1995).

(3) genes that enhance insect resistance in the seed (for example, resistance to weevils). Suitable genes include the α -amylase inhibitor gene which kills seed weevils (Schmidt, 1994).

REFERENCES

5 Ainley et al. (1993) Regulatable endogenous production of cytokinins up to "toxic" levels in transgenic plants and plant tissues. *Plant Mol. Biol.* **22**:13-23.

10 Altschul et al. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**:403-410.

15 Altschul et al. (1994) *Nature Genet.*, **6**, 119-29.

20 An et al. (1988) *Plant Physiol.* **88**:547.

Ausubel et al. (1987) In *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Intersciences.

25 Badenoch-Jones et al. (1996) Effect of cytokinins supplied via the xylem at multiples of endogenous concentrations on transcription and senescence in derooted seedlings of oat and wheat. *Plant Cell and Environment* **19**:504-516.

30 Benfey & Chua (1990) The cauliflower mosaic virus 35S promoter: Combinatorial regulation of transcription in plants. *Science* **250**:959-966.

Birch et al. (1997) Plant transformation: Problems and strategies for practical application. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**:297-326.

35 Bolton & McCarthy (1962) *Proc. Natl. Acad. Sci. USA* **48**:1390.

Bonner et al. (1973) *J. Mol. Biol.* **81**:123.

40 Brzobohaty et al. (1993) Release of active cytokinin by a β -glucosidase localized to the maize root meristem. *Science* **262**:1051-1054.

Bustos et al. (1989) *Plant Cell* **1**:839.

45 Callis et al. (1988) *Plant Physiol.* **88**:965.

50 Carpenter et al. (1992) Preferential expression of an α -tubulin gene of *Arabidopsis* in pollen. *The Plant Cell* **4**:557-571.

55 Chang et al. (1986) *Saccharomyces cerevisiae* secretes and correctly processes human interferon hybrid protein containing yeast invertase signal peptides. *Mol. And Cell. Biol.* **6**:1812-1819.

Corpet et al. (1988) *Nucleic Acids Research* **16**, 10881-90.

Denis et al. (1993) Expression of engineered nuclear male sterility in *Brassica napus*. *Plant Physiol.* **101**:1295-1304.

Dixon et al. (1989) Zeatin glycosylation enzymes in *Phaseolus*. Isolation of O-glucosyltransferase from *P. lunatus* and comparison to O-xylosyltransferase from *P. vulgaris*. *Plant Physiology* **90**:1316-1321.

Doyle & Doyle (1990) Isolation of plant DNA from fresh tissue. *Focus* **12**:13-15.

Fromm et al. (1989) *Plant Cell* **1**:977.

Gan & Amansino (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270**:1986-1988.

5 Gatz et al. (1997) Chemical control of gene expression. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48**:89-108.

Gelvin et al. (1990) *Plant Molecular Biology Manual*, Kluwer Academic Publishers.

10 Gilmartin et al. (1992) Characterization of a gene encoding a DNA binding protein with specificity for a light-responsive element. *The Plant Cell* **4**:839-949.

Harlow & Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York.

15 Higgins & Sharp (1988) *Gene*, **73**: 237-244.

Higgins & Sharp (1989) *CABIOS* **5**: 151-153.

20 Hill et al. (1994) in *Selenium in Biology in Human Health*, ed. Burk, R. F. (Springer, New York), pp. 117-132.

Hradecka & Petr (1992) The effect of cytokinins on the yield of some cereal plants. In: *Physiology and Biochemistry of Cytokinins in Plants*. Eds. Kaminek, M., DWS. Mok and E. Zazimalova. SPB Academic Publishing bv. 1992. pp. 245-247.

25 Huang et al. (1992) *Computer Applications in the Biosciences* **8**, 155-65

Innis et al. (1990) *PCR Protocols, A Guide to Methods and Applications*, Innis et al. (eds.), Academic Press, Inc., San Diego, California.

30 Jameson (1994) Cytokinin metabolism and compartmentation. In: *Cytokinins, Chemistry, Activity and Function*. Eds. Mok, DWS and MC Mok. CRC Press. p. 113-128.

35 Jorgensen et al. (1998) An RNA-based information super highway in plants. *Science* **279**:1486-1487.

Karanov et al. (1992) Physiology and application of phenylurea cytokinins. In: *Progress in Plant Growth Regulation* (C.M. Karssen et al., eds.), pp. 842-851.

40 Kawasaki et al. (1990). In *PCR Protocols, A Guide to Methods and Applications*, Innis et al. (eds.), 21-27, Academic Press, Inc., San Diego, California.

Krikorian (1995) Hormones in tissue culture and micropropagation. In: *Plant Hormones-Physiology, Biochemistry and Molecular Biology*. 2nd Edition. Ed. Davies, PJ. Kluwer Academic Publishers. pp. 774-796.

45 Kuhlemeier et al. (1989) *Plant Cell* **1**: 471.

50 Letham (1963) Zeatin, a factor inducing cell division from *Zea mays*. *Life Sciences* **8**:569-573.

Letham et al. (1976) O-Glucosylzeatin and related compounds - a new group of cytokinin metabolites. *Ann. Botany* **41**:261-263.

55 Lewis, et al. (1996) Cytokinins and fruit development in the kiwifruit (*Actinidia deliciosa*).

II. Effects of reduced pollination and CPPU application. *Physiol. Plant.* **98**:187-195.

Liang & Richardson (1993) Expression and characterization of human lactoferrin in yeast (*Saccharomyces cerevisiae*). *J. Agric. Food Chem.* **41**:1800-1807.

5 Marcotte et al. (1989) *Plant Cell* **1**:969.

Martin et al. (1990) Monoclonal antibody specific to zeatin O-glycosyltransferases of *Phaseolus*. *Plant Physiology* **94**:1290-1294.

10 McGaw et al. (1983) Cytokinin oxidase from *Zea mays* kernels and *Vinca rosea* crown-gall tissue. *Planta* **159**:30-37.

15 Meyer & Saedler (1996) Homology dependent gene silencing in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47**:23-48.

Meyers & Miller (1988) *Computer Applic. Biol. Sci.*, **4**: 11-17.

20 Miller et al. (1956) Isolation, structure and synthesis of kinetin, a substance promoting cell division. *Journal of American Chemical Society* **78**:1375-1380.

Miller et al. (1955) Kinetin, a cell division factor from deoxyribonucleic acid. *Journal of American Chemical Society* **77**:1392.

25 Minana et al. (1989) Endogenous cytokinins in developing fruits of seeded and seedless citrus cultivars. *J. Exp. Bot.* **219**:1127-1134.

Mok & Martin (1994) Cytokinin metabolic enzymes. In: *Cytokinins, Chemistry, Activity and Function*. Eds. Mok, DWS and MC Mok. CRC Press. p. 129-137.

30 Mok (1994) Cytokinins and plant development - An overview. In: *Cytokinins, Chemistry, Activity and Function*. Eds. Mok, DWS and MC Mok. CRC Press. p. 155-166.

Molvig et al. (1997) Enhanced methionine levels and increased nutritive value of seeds of transgenic lupins (*Lupinus angustifolius* L.) expressing a sunflower seed albumin gene. *Proc. Natl. Acad. Sci.* **94**:8393-8398.

35 Needelman & Wunsch (1970) *J. Mol. Biol.* **48**: 443.

40 Newman et al. (1994) *Plant Physiol.* **106**: 1241-1255.

Ochman et al. (1989) Inverse polymerase chain reaction. In: *PCR Technology-Principles and Applications for DNA Amplification*. Ed. Erlich, HA. pp. 105-111.

45 Odel et al. (1985) *Nature* **313**:810.

Odell et al. (1994) Seed specific gene activation mediated by the Cre/lox site-specific recombination system. *Plant Physiol.* **106**:447-458.

50 Opperman et al. (1993) Root knot nematode directed expression of a plant root specific gene. *Science* **263**:221-223.

Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* **85**: 2444.

55 Pearson et al. (1994) *Methods in Molecular Biology* **24**, 307-31.

Pouwels et al. (1987) *Cloning Vectors: A Laboratory Manual*, 1985, supp.

Roshal et al. (1987). *EMBO J.* **6**:1155.

5 Saalbach et al. (1996) The vacuolar targeting signal of the 2S albumin from Brazil nut resides at the C terminus and involves the C-terminal propeptide as an essential element. *Plant Physiol.* **112**:975-985.

10 Saiki et al. (1989). *Proc. Nat. Acad. Sci. USA* **86**:6230-6234.

Sambrook et al. (1989) In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York.

15 Schaffner & Sheen (1991) *Plant Cell* **3**:997.

Schernthaner et al. (1988) *EMBO J.* **7**:1249.

Schmidt (1994) Genetic engineering yields. First pest resistant seeds. *Science* **265**:739.

20 Shaw et al. (1964) A synthesis of zeatin. *Proceedings of Chemical Society* **231**.

Shaw (1994) Chemistry of adenine cytokinins. In: *Cytokinins, Chemistry, Activity and Function*. Eds. Mok, DWS and MC Mok. CRC Press. p. 15-34.

25 Shewry et al. (1995) Seed storage proteins: structures and biosynthesis. *The Plant Cell* **7**:945-956.

Siebertz et al. (1989) *Plant Cell* **1**:961.

30 Skoog et al. (1965) Cytokinins. *Science* **148**:532.

Smith et al. (1985) Heterologous protein secretion from yeast. *Science* **229**:1219-1224.

35 Smith & Waterman (1981) *Adv. Appl. Math.* **2**: 482.

Southern (1975). *J. Mol. Biol.* **98**:503.

40 Southern et al. (1982) *J. Mol. Appl. Genet.* **1**:327-341.

Stockhause et al. (1997) The promoter of the gene encoding the C₄ form of phosphoenolpyruvate carboxylase directs mesophyll-specific expression in transgenic C₄ *Flaveria* spp. *The Plant Cell* **9**:479-489.

45 Terada & Shimamoto (1990) *Mol. Gen. Genet.* **220**:389.

Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York.

50 Turner, J. E., Mok, D.W.S., Mok, M. C. and Shaw, G. 1987. Proceedings of National Academy of Sciences (USA). **84**: 3714-717.

Weissbach & Weissbach (1989) Methods for Plant Molecular Biology, Academic Press.

55 Worley et al. (1995) BEAUTY: an enhanced BLAST-based search tool that integrates

-39-

multiple biological information resources into sequence similarity search results.
Genome Research 5:173-184.

We claim:

1. A purified polypeptide comprising an amino acid sequence selected from the group consisting of:

5 (a) the sequence set forth in Seq. I.D. No. 4; and
(b) the sequence set forth in Seq. I.D. No. 12.

2. A nucleic acid molecule encoding a polypeptide according to claim 1.

10 3. A recombinant nucleic acid molecule comprising a promoter sequence operably linked to a nucleic acid molecule according to claim 2.

4. A transgenic plant comprising a recombinant nucleic acid molecule according to claim 3.

15 5. A purified polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) the sequence set forth in Seq. I.D. No. 4;
(b) sequences that differ from the sequence defined in (a) by one or more

20 conservative amino acid substitutions; and
(c) sequences that share at least 70% sequence identity with the sequence defined in (a),
wherein the polypeptide has zeatin-O-glucosyltransferase activity.

25 6. An isolated nucleic acid molecule that encodes a polypeptide according to claim 5.

7. A recombinant nucleic acid molecule comprising a promoter sequence operably linked to a nucleic acid molecule according to claim 6.

30 8. A transgenic plant comprising a recombinant nucleic acid molecule according to claim 7.

9. A purified polypeptide comprising an amino acid sequence selected from the 35 group consisting of:

(a) the sequence set forth in Seq. I.D. No. 12;
(b) sequences that differ from the sequence defined in (a) by one or more

conservative amino acid substitutions; and

(c) sequences that share at least 70% sequence identity with the sequence defined in (a),

wherein the polypeptide has zeatin-O-xylosyltransferase activity.

5

10. An isolated nucleic acid molecule that encodes a polypeptide according to claim 9.

11. A recombinant nucleic acid molecule comprising a promoter sequence operably

10 linked to a nucleic acid molecule according to claim 10.

12. A transgenic plant comprising a recombinant nucleic acid molecule according to claim 11.

15 13. An isolated nucleic acid molecule comprising at least 15 consecutive nucleotides of a sequence selected from the group consisting of:

(a) Seq. I.D. No. 3; and

(b) Seq. I.D. No. 11.

20 14. An isolated nucleic acid molecule according to claim 13 wherein the nucleic acid molecule comprises at least 30 consecutive nucleotides of the selected sequence.

15. An isolated nucleic acid molecule according to claim 14 wherein the nucleic acid molecule comprises at least 50 consecutive nucleotides of the selected sequence.

25

16. A recombinant nucleic acid molecule comprising a promoter sequence operably linked to a nucleic acid molecule according to claim 13.

30 17. A transgenic plant comprising a recombinant nucleic acid molecule according to claim 16.

18. A recombinant nucleic acid molecule according to claim 16 wherein the nucleic acid molecule is in antisense orientation with respect to the promoter sequence.

35 19. A transgenic plant comprising a recombinant nucleic acid molecule according to claim 18.

-42-

20. An isolated oligonucleotide comprising at least 15 consecutive nucleotides of a nucleic acid sequence selected from the group consisting of:

- (a) Seq. I.D. No. 1;
- (b) Seq. I.D. No. 2;
- 5 (c) Seq. I.D. No. 3;
- (d) Seq. I.D. No. 11; and

21. An isolated nucleic acid molecule according to claim 20 wherein the nucleic acid molecule comprises at least 30 consecutive nucleotides of the selected sequence.

10

22. An isolated nucleic acid molecule according to claim 21 wherein the nucleic acid molecule comprises at least 50 consecutive nucleotides of the selected sequence.

15 23. A recombinant nucleic acid molecule comprising a promoter sequence operably linked to a nucleic acid sequence, wherein the promoter sequence comprises a zeatin-O-glucosyltransferase promoter.

20 24. A recombinant nucleic acid molecule according to claim 23 wherein the promoter sequence comprises at least 50 consecutive nucleotides of the sequence shown in Seq. I.D. No. 6.

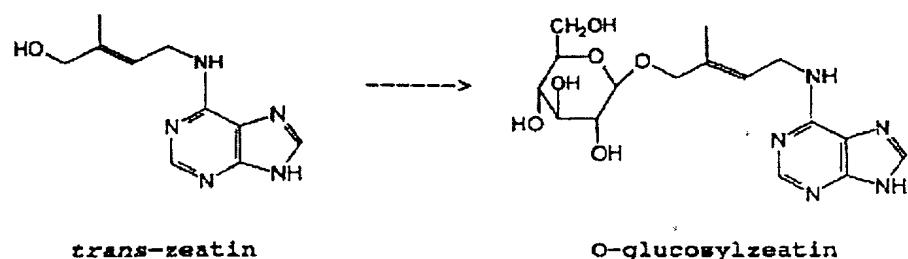


FIG. 1

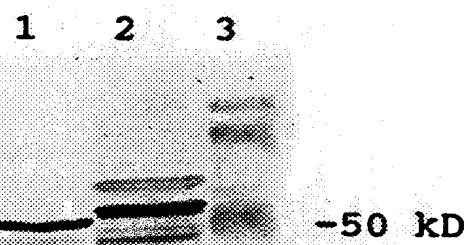
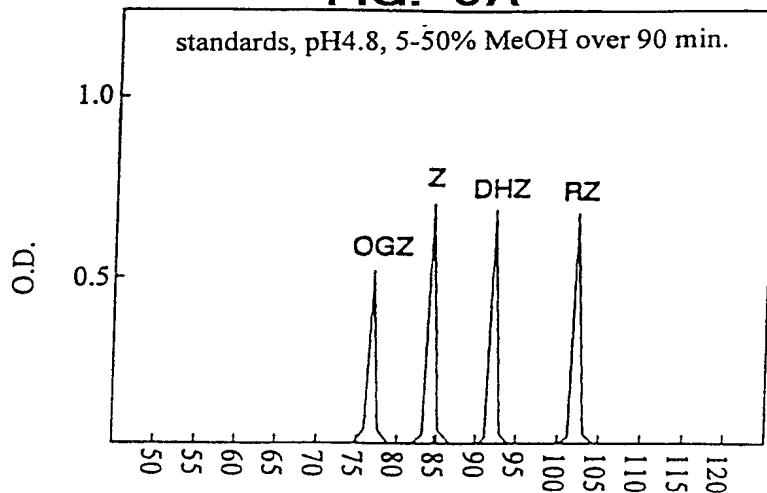
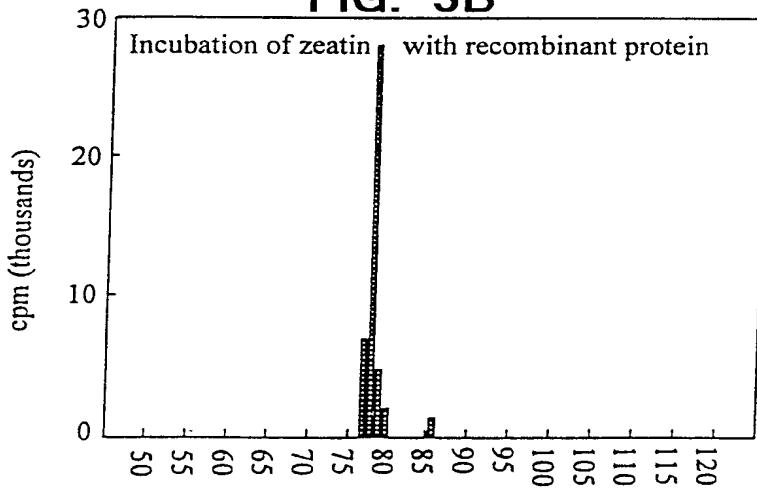
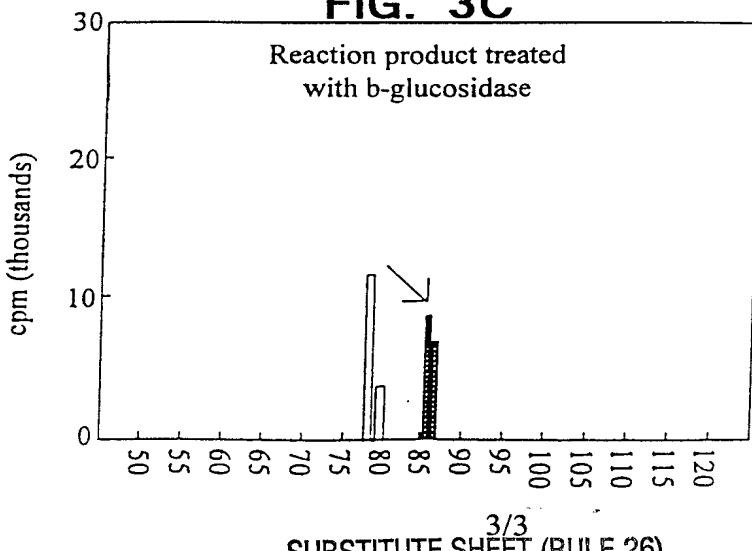


FIG. 2

FIG. 3A**FIG. 3B****FIG. 3C**

SEQUENCE LISTING

<110> Mok, David

<120> Enzymes Responsible for the Metabolism of Zeatin

<130> 51524

<140>

<141>

<150> 60/080852

<151> 1998-04-06

<160> 16

<170> PatentIn Ver. 2.0

<210> 1

<211> 1523

<212> DNA

<213> Phaseolus lunatus

<220>

<221> CDS

<222> (33)..(1409)

<400> 1

gaagcatagc atcttgctac ccaaattcac ca atg gct ttg aat gac aaa agc 53
Met Ala Leu Asn Asp Lys Ser
1 5

att cct cat gaa acc aaa gtg gtg ctt ttg ata cct ttc cct gca 101
Ile Pro His Glu Thr Lys Val Val Val Leu Leu Ile Pro Phe Pro Ala
10 15 20

caa ggt cac ctc aac cag ttt ctg cac cta tct cgc tta atc gtg gca 149
Gln Gly His Leu Asn Gln Phe Leu His Leu Ser Arg Leu Ile Val Ala
25 30 35

caa aac ata cca gtc cat tat gtt ggc act gtc aca cac att cgc cag 197
Gln Asn Ile Pro Val His Tyr Val Gly Thr Val Thr His Ile Arg Gln
40 45 50 55

gca aca ctt cga tac aac aac cct act tca aac atc cat ttc cat gcc 245
Ala Thr Leu Arg Tyr Asn Asn Pro Thr Ser Asn Ile His Phe His Ala
60 65 70

ttt caa gtt cca ccc ttt gtt tcc cct ccc aat cca gaa gac gat 293
Phe Gln Val Pro Pro Phe Val Ser Pro Pro Pro Asn Pro Glu Asp Asp
75 80 85

ttc cca tct cat cta att cct tcc ttt gag gcc tct gca cac ctt cgg 341
Phe Pro Ser His Leu Ile Pro Ser Phe Glu Ala Ser Ala His Leu Arg
90 95 100

gag ccc gtg ggg aaa ctt ctt caa tcc ctc tca tca caa gcc aaa agg 389
Glu Pro Val Gly Lys Leu Leu Gln Ser Leu Ser Ser Gln Ala Lys Arg
105 110 115

gtc gta gtc atc aat gac tcc ctc atg gca tct gtg gca caa gat gcc 437

Val	Val	Val	Ile	Asn	Asp	Ser	Leu	Met	Ala	Ser	Val	Ala	Gln	Asp	Ala	
120																135
gca aac atc tca aat gtt gaa aac tac act ttt cac agc ttc tct gcc															485	
Ala	Asn	Ile	Ser	Asn	Val	Glu	Asn	Tyr	Thr	Phe	His	Ser	Phe	Ser	Ala	
																140
140 145 150																
ttt aat acc tcc ggt gat ttt tgg gaa gaa atg gga aag ccc ccg gtt															533	
Phe	Asn	Thr	Ser	Gly	Asp	Phe	Trp	Glu	Glu	Met	Gly	Lys	Pro	Pro	Val	
																155
155 160 165																
gga gat ttc cat ttc cca gaa ttt cct tct ctt gaa gga tgc atc gca															581	
Gly	Asp	Phe	His	Phe	Pro	Glu	Phe	Pro	Ser	Leu	Glu	Gly	Cys	Ile	Ala	
																170
170 175 180																
gcc cag ttc aag ggc ttt cgt act gca cag tat gaa ttc cgc aaa ttc															629	
Ala	Gln	Phe	Lys	Gly	Phe	Arg	Thr	Ala	Gln	Tyr	Glu	Phe	Arg	Lys	Phe	
																185
185 190 195																
aac aat ggc gat att tac aac acc agc agg gtg att gaa ggt cct tac															677	
Asn	Asn	Gly	Asp	Ile	Tyr	Asn	Thr	Ser	Arg	Val	Ile	Glu	Gly	Pro	Tyr	
																200
200 205 210 215																
gtt gag ttg ctg gag ctt ttc aat ggc ggc aag aag gtt tgg gca ttg															725	
Val	Glu	Leu	Leu	Glu	Leu	Phe	Asn	Gly	Gly	Lys	Val	Trp	Ala	Leu		
																220
220 225 230																
ggg cca ttt aac cct tta gcc gtt gag aag aaa gat tca ata gga ttt															773	
Gly	Pro	Phe	Asn	Pro	Leu	Ala	Val	Glu	Lys	Lys	Asp	Ser	Ile	Gly	Phe	
																235
235 240 245																
agg cac cca tgc atg gag tgg ctt gat aaa caa gag cca agt tca gtc															821	
Arg	His	Pro	Cys	Met	Glu	Trp	Leu	Asp	Lys	Gln	Glu	Pro	Ser	Ser	Val	
																250
250 255 260																
ata tat ata tcc ttc ggg acc acg aca gct ttg aga gat gaa caa atc															869	
Ile	Tyr	Ile	Ser	Phe	Gly	Thr	Thr	Thr	Ala	Leu	Arg	Asp	Glu	Gln	Ile	
																265
265 270 275																
caa cag ata gca act ggg ttg gaa caa agc aag cag aag ttc atc tgg															917	
Gln	Gln	Ile	Ala	Thr	Gly	Leu	Glu	Gln	Ser	Lys	Gln	Lys	Phe	Ile	Trp	
																280
280 285 290 295																
gtg ctg aga gaa gcc gat aaa ggg gac atc ttt gcc gga agt gaa gca															965	
Val	Leu	Arg	Glu	Ala	Asp	Lys	Gly	Asp	Ile	Phe	Ala	Gly	Ser	Glu	Ala	
																300
300 305 310																
aaa agg tat gaa ctt cca aag ggt ttt gag gag aga gtg gaa gga atg															1013	
Lys	Arg	Tyr	Glu	Leu	Pro	Lys	Gly	Phe	Glu	Arg	Val	Glu	Gly	Met		
																315
315 320 325																
ggg ctg gtt gtg agg gac tgg gca ccc caa ttg gaa att ctg agc cac															1061	
Gly	Leu	Val	Val	Arg	Asp	Trp	Ala	Pro	Gln	Leu	Glu	Ile	Leu	Ser	His	
																330
330 335 340																
agt tca aca ggg ggg ttt atg agc cat tgt gga tgg aac tcg tgc ttg															1109	
Ser	Ser	Thr	Gly	Gly	Phe	Met	Ser	His	Cys	Gly	Trp	Asn	Ser	Cys	Leu	
																345
345 350 355																
gag agc ata acc atg ggg gtg cca ata gca aca tgg ccc atg cac tct															1157	
Glu	Ser	Ile	Thr	Met	Gly	Val	Pro	Ile	Ala	Thr	Trp	Pro	Met	His	Ser	

360	365	370	375	
gac cag cca aga aat gca gtt ttg gtt aca gag gtt ctg aag gtt ggt				1205
Asp	Gln	Pro	Arg	Asn Ala Val Leu Val Thr Glu Val Leu Lys Val Gly
380				390
ttg gtt gtg aag gat tgg gca cag agg aat tcg ttg gtg agt gct tca				1253
Leu	Val	Val	Lys	Asp Trp Ala Gln Arg Asn Ser Leu Val Ser Ala Ser
395				400
gtt gtt gag aat ggt gtg aga agg ttg atg gaa aca aag gaa ggt gat				1301
Val	Val	Glu	Asn	Gly Val Arg Arg Leu Met Glu Thr Lys Glu Gly Asp
410				415
gag atg aga cag aga gca gtg agg ctt aaa aat gcc atc cat agg tca				1349
Glu	Met	Arg	Gln	Arg Ala Val Arg Leu Lys Asn Ala Ile His Arg Ser
425				430
atg gat gaa ggt gga gtt tct cac atg gag atg ggt tct ttc att gca				1397
Met	Asp	Glu	Gly	Gly Val Ser His Met Glu Met Gly Ser Phe Ile Ala
440				445
cac atc tct aaa tagtcataacc atttagcctg ttcttatagc aaaatatgac				1449
His	Ile	Ser	Lys	
agaagtttagt tgtcatcatg ttgttgatgc aaaataattt cctatgacat tatccaataa 1509				
taaagcttct gttg 1523				

<210> 2
 <211> 1632
 <212> DNA
 <213> Phaseolus lunatus

<220>
 <221> CDS
 <222> (142)..(1518)

aagctttaa ataagaaata tttttgcta tatataaattc ttgagtataa aatgtatgtt			60												
catacttgat ctactaccaa cttaagcata agcagccagt gtcaaagttg aagcatagca			120												
tcttgctacc caaattcacc a atg gct ttg aat gac aaa agc att cct cat			171												
Met	Ala	Leu	Asn	Asp	Lys	Ser	Ile	Pro	His						
1					5				10						
gaa acc aaa gtg gtg ctt ttg ata cct ttc cct gca caa ggt cac			219												
Glu	Thr	Lys	Val	Val	Leu	Leu	Ile	Pro	Phe	Pro	Ala	Gln	Gly	His	
15						20				25					
ctc aac cag ttt ctg cac cta tct cgc tta atc gtg gca caa aac ata			267												
Leu	Asn	Gln	Phe	Leu	His	Leu	Ser	Arg	Leu	Ile	Val	Ala	Gln	Asn	Ile
30						35				40					
cca gtc cat tat gtt ggc act gtc aca cac att cgc cag gca aca ctt			315												
Pro	Val	His	Tyr	Val	Gly	Thr	Val	Thr	His	Ile	Arg	Gln	Ala	Thr	Leu
45						50				55					
cga tac aac aac cct act tca aac atc cat ttc cat gcc ttt caa gtt			363												

Arg	Tyr	Asn	Asn	Pro	Thr	Ser	Asn	Ile	His	Phe	His	Ala	Phe	Gln	Val	
60						65					70					
cca	ccc	ttt	gtt	tcc	cct	cct	ccc	aat	cca	gaa	gac	gat	ttc	cca	tct	411
Pro	Pro	Phe	Val	Ser	Pro	Pro	Pro	Asn	Pro	Glu	Asp	Asp	Phe	Pro	Ser	
75					80					85					90	
cat	cta	att	cct	tcc	ttt	gag	gcc	tct	gca	cac	ctt	cgg	gag	ccc	gtg	459
His	Leu	Ile	Pro	Ser	Phe	Glu	Ala	Ser	Ala	His	Leu	Arg	Glu	Pro	Val	
						95				100					105	
ggg	aaa	ctt	ctt	caa	tcc	ctc	tca	tca	caa	gcc	aaa	agg	gtc	gta	gtc	507
Gly	Lys	Leu	Leu	Gln	Ser	Leu	Ser	Ser	Gln	Ala	Lys	Arg	Val	Val	Val	
						110				115					120	
atc	aat	gac	tcc	ctc	atg	gca	tct	gtg	gca	caa	gat	gcc	gca	aac	atc	555
Ile	Asn	Asp	Ser	Leu	Met	Ala	Ser	Val	Ala	Gln	Asp	Ala	Ala	Asn	Ile	
						125				130					135	
tca	aat	gtt	gaa	aac	tac	act	ttt	cac	agc	ttc	tct	gcc	ttt	aat	acc	603
Ser	Asn	Val	Glu	Asn	Tyr	Thr	Phe	His	Ser	Phe	Ser	Ala	Phe	Asn	Thr	
						140				145					150	
tcc	ggt	gat	ttt	tgg	gaa	gaa	atg	gga	aag	ccc	ccg	gtt	gga	gat	ttc	651
Ser	Gly	Asp	Phe	Trp	Glu	Glu	Met	Gly	Lys	Pro	Pro	Val	Gly	Asp	Phe	
						155				160					170	
cat	ttc	cca	gaa	ttt	cct	tct	ctt	gaa	gga	tgc	atc	gca	gcc	cag	ttc	699
His	Phe	Pro	Glu	Phe	Pro	Ser	Leu	Glu	Gly	Cys	Ile	Ala	Ala	Gln	Phe	
						175				180					185	
aag	ggc	ttt	cgt	act	gca	cag	tat	gaa	ttc	cgc	aaa	ttc	aac	aat	ggc	747
Lys	Gly	Phe	Arg	Thr	Ala	Gln	Tyr	Glu	Phe	Arg	Lys	Phe	Asn	Asn	Gly	
						190				195					200	
gat	att	tac	aac	acc	agc	agg	gtg	att	gaa	ggt	cct	tac	gtt	gag	ttg	795
Asp	Ile	Tyr	Asn	Thr	Ser	Arg	Val	Ile	Glu	Gly	Pro	Tyr	Val	Glu	Leu	
						205				210					215	
ctg	gag	ctt	aat	ggc	ggc	aag	aag	gtt	tgg	gca	ttg	ggg	cca	ttt		843
Leu	Glu	Leu	Phe	Asn	Gly	Gly	Lys	Lys	Val	Trp	Ala	Leu	Gly	Pro	Phe	
						220				225					230	
aac	cct	tta	gcc	gtt	gag	aag	aaa	gat	tca	ata	gga	ttt	agg	cac	cca	891
Asn	Pro	Leu	Ala	Val	Glu	Lys	Lys	Asp	Ser	Ile	Gly	Phe	Arg	His	Pro	
						235				240					250	
tgc	atg	gag	tgg	ctt	gat	aaa	caa	gag	cca	agt	tca	gtc	ata	tat	ata	939
Cys	Met	Glu	Trp	Leu	Asp	Lys	Gln	Glu	Pro	Ser	Ser	Val	Ile	Tyr	Ile	
						255				260					265	
tcc	ttc	ggg	acc	acg	aca	gct	ttg	aga	gat	gaa	caa	atc	caa	cag	ata	987
Ser	Phe	Gly	Thr	Thr	Ala	Leu	Arg	Asp	Glu	Gln	Ile	Gln	Gln	Ile		
						270				275					280	
gca	act	ggg	ttg	gaa	caa	agc	aag	cag	aag	ttc	atc	tgg	gtg	ctg	aga	1035
Ala	Thr	Gly	Leu	Glu	Gln	Ser	Lys	Gln	Lys	Phe	Ile	Trp	Val	Leu	Arg	
						285				290					295	
gaa	gcc	gat	aaa	ggg	gac	atc	ttt	gcc	gga	agt	gaa	gca	aaa	agg	tat	1083
Glu	Ala	Asp	Lys	Gly	Asp	Ile	Phe	Ala	Gly	Ser	Glu	Ala	Lys	Arg	Tyr	

300	305	310													
gaa ctt cca aag ggt ttt gag gag aga gtg gaa gga atg ggg ctg gtt 1131															
Glu	Leu	Pro	Lys	Gly	Phe	Glu	Glu	Arg	Val	Glu	Gly	Met	Gly	Leu	Val
315		320		325		325		330		330		330		330	
gtg agg gac tgg gca ccc caa ttg gaa att ctg agc cac agt tca aca 1179															
Val	Arg	Asp	Trp	Ala	Pro	Gln	Leu	Glu	Ile	Leu	Ser	His	Ser	Ser	Thr
335		340		340		345		345		345		345		345	
ggg ggg ttt atg agc cat tgt gga tgg aac tcg tgc ttg gag agc ata 1227															
Gly	Gly	Phe	Met	Ser	His	Cys	Gly	Trp	Asn	Ser	Cys	Leu	Glu	Ser	Ile
350		355		355		360		360		360		360		360	
acc atg ggg gtg cca ata gca aca tgg ccc atg cac tct gac cag cca 1275															
Thr	Met	Gly	Val	Pro	Ile	Ala	Thr	Trp	Pro	Met	His	Ser	Asp	Gln	Pro
365		370		370		375		375		375		375		375	
aga aat gca gtt ttg gtt aca gag gtt ctg aag gtt ggt ttg gtt gtg 1323															
Arg	Asn	Ala	Val	Leu	Val	Thr	Glu	Val	Leu	Lys	Val	Gly	Leu	Val	Val
380		385		385		390		390		390		390		390	
aag gat tgg gca cag agg aat tcg ttg gtg agt gct tca gtt gtt gag 1371															
Lys	Asp	Trp	Ala	Gln	Arg	Asn	Ser	Leu	Val	Ser	Ala	Ser	Val	Val	Glu
395		400		400		405		405		405		405		405	
aat ggt gtg aga agg ttg atg gaa aca aag gaa ggt gat gag atg aga 1419															
Asn	Gly	Val	Arg	Arg	Leu	Met	Glu	Thr	Lys	Glu	Gly	Asp	Glu	Met	Arg
415		420		420		425		425		425		425		425	
cag aga gca gtg agg ctt aaa aat gcc atc cat agg tca atg gat gaa 1467															
Gln	Arg	Ala	Val	Arg	Leu	Lys	Asn	Ala	Ile	His	Arg	Ser	Met	Asp	Glu
430		435		435		440		440		440		440		440	
ggg gga gtt tct cac atg gag atg ggt tct ttc att gca cac atc tct 1515															
Gly	Gly	Val	Ser	His	Met	Glu	Met	Gly	Ser	Phe	Ile	Ala	His	Ile	Ser
445		450		450		455		455		455		455		455	
aaa tagtcataacc atttagcctg ttcttatagc aaaatatgac agaagtttagt 1568															
Lys															
tgtcatcatg ttgttgatgc aaaataattt cctatgacat tatccaataa taaagcttct 1628															
gttg															
1632															

<210> 3
<211> 1377
<212> DNA
<213> Phaseolus lunatus

<220>
<221> CDS
<222> (1)..(1377)

<400> 3
atg gct ttg aat gac aaa agc att cct cat gaa acc aaa gtg gtg gtg 48
Met Ala Leu Asn Asp Lys Ser Ile Pro His Glu Thr Lys Val Val Val
1 5 10 15

ctt ttg ata cct ttc cct gca caa ggt cac ctc aac cag ttt ctg cac 96

Leu	Leu	Ile	Pro	Phe	Pro	Ala	Gln	Gly	His	Leu	Asn	Gln	Phe	Leu	His	
20										25				30		
cta	tct	cgc	tta	atc	gtg	gca	caa	aac	ata	cca	gtc	cat	tat	gtt	ggc	144
Leu	Ser	Arg	Leu	Ile	Val	Ala	Gln	Asn	Ile	Pro	Val	His	Tyr	Val	Gly	
35													45			
act	gtc	aca	cac	att	cgc	cag	gca	aca	ctt	cga	tac	aac	aac	cct	act	192
Thr	Val	Thr	His	Ile	Arg	Gln	Ala	Thr	Leu	Arg	Tyr	Asn	Asn	Pro	Thr	
50													60			
tca	aac	atc	cat	ttc	cat	gcc	ttt	caa	gtt	cca	ccc	ttt	gtt	tcc	cct	240
Ser	Asn	Ile	His	Phe	His	Ala	Phe	Gln	Val	Pro	Pro	Phe	Val	Ser	Pro	
65													80			
cct	ccc	aat	cca	gaa	gac	gat	ttc	cca	tct	cat	cta	att	cct	tcc	ttt	288
Pro	Pro	Asn	Pro	Glu	Asp	Asp	Phe	Pro	Ser	His	Leu	Ile	Pro	Ser	Phe	
85													95			
gag	gcc	tct	gca	cac	ctt	cgg	gag	ccc	gtg	ggg	aaa	ctt	ctt	caa	tcc	336
Glu	Ala	Ser	Ala	His	Leu	Arg	Glu	Pro	Val	Gly	Lys	Leu	Leu	Gln	Ser	
100													110			
ctc	tca	tca	caa	gcc	aaa	agg	gtc	gta	gtc	atc	aat	gac	tcc	ctc	atg	384
Leu	Ser	Ser	Gln	Ala	Lys	Arg	Val	Val	Val	Ile	Asn	Asp	Ser	Leu	Met	
115													125			
gca	tct	gtg	gca	caa	gat	gcc	gca	aac	atc	tca	aat	gtt	gaa	aac	tac	432
Ala	Ser	Val	Ala	Gln	Asp	Ala	Ala	Asn	Ile	Ser	Asn	Val	Glu	Asn	Tyr	
130													140			
act	ttt	cac	agc	ttc	tct	gcc	ttt	aat	acc	tcc	ggt	gat	ttt	tgg	gaa	480
Thr	Phe	His	Ser	Phe	Ser	Ala	Phe	Asn	Thr	Ser	Gly	Asp	Phe	Trp	Glu	
145													160			
gaa	atg	gga	aag	ccc	ccg	gtt	gga	gat	ttc	cat	ttc	cca	gaa	ttt	cct	528
Glu	Met	Gly	Lys	Pro	Pro	Val	Gly	Asp	Phe	His	Phe	Pro	Glu	Phe	Pro	
165													175			
tct	ctt	gaa	gga	tgc	atc	gca	gcc	cag	ttc	aag	ggc	ttt	cgt	act	gca	576
Ser	Leu	Glu	Gly	Cys	Ile	Ala	Ala	Gln	Phe	Lys	Gly	Phe	Arg	Thr	Ala	
180													190			
cag	tat	gaa	ttc	cgc	aaa	ttc	aac	aat	ggc	gat	att	tac	aac	acc	agc	624
Gln	Tyr	Glu	Phe	Arg	Lys	Phe	Asn	Asn	Gly	Asp	Ile	Tyr	Asn	Thr	Ser	
195													205			
agg	gtg	att	gaa	ggt	cct	tac	gtt	gag	ttg	ctg	gag	ctt	tcc	aat	ggc	672
Arg	Val	Ile	Glu	Gly	Pro	Tyr	Val	Glu	Leu	Leu	Glu	Leu	Phe	Asn	Gly	
210													220			
ggc	aag	aag	gtt	tgg	gca	ttg	ggg	cca	ttt	aac	cct	tta	gcc	gtt	gag	720
Gly	Lys	Lys	Val	Trp	Ala	Leu	Gly	Pro	Phe	Asn	Pro	Leu	Ala	Val	Glu	
225													235		240	
aag	aaa	gat	tca	ata	gga	ttt	agg	cac	cca	tgc	atg	gag	tgg	ctt	gat	768
Lys	Lys	Asp	Ser	Ile	Gly	Phe	Arg	His	Pro	Cys	Met	Glu	Trp	Leu	Asp	
245													255			
aaa	caa	gag	cca	agt	tca	gtc	ata	tat	ata	tcc	ttc	ggg	acc	acg	aca	816
Lys	Gln	Glu	Pro	Ser	Ser	Val	Ile	Tyr	Ile	Ser	Phe	Gly	Thr	Thr	Thr	
225																

260	265	270	
gct ttg aga gat gaa caa atc caa cag ata gca act ggg ttg gaa caa Ala Leu Arg Asp Glu Gln Ile Gln Gln Ile Ala Thr Gly Leu Glu Gln			864
275	280	285	
agc aag cag aag ttc atc tgg gtg ctg aga gaa gcc gat aaa ggg gac Ser Lys Gln Lys Phe Ile Trp Val Leu Arg Glu Ala Asp Lys Gly Asp			912
290	295	300	
atc ttt gcc gga agt gaa gca aaa agg tat gaa ctt cca aag ggt ttt Ile Phe Ala Gly Ser Glu Ala Lys Arg Tyr Glu Leu Pro Lys Gly Phe			960
305	310	315	320
gag gag aga gtg gaa gga atg ggg ctg gtt gtg agg gac tgg gca ccc Glu Glu Arg Val Glu Gly Met Gly Leu Val Val Arg Asp Trp Ala Pro			1008
325	330	335	
caa ttg gaa att ctg agc cac agt tca aca ggg ggg ttt atg agc cat Gln Leu Glu Ile Leu Ser His Ser Ser Thr Gly Gly Phe Met Ser His			1056
340	345	350	
tgt gga tgg aac tcg tgc ttg gag agc ata acc atg ggg gtg cca ata Cys Gly Trp Asn Ser Cys Leu Glu Ser Ile Thr Met Gly Val Pro Ile			1104
355	360	365	
gca aca tgg ccc atg cac tct gac cag cca aga aat gca gtt ttg gtt Ala Thr Trp Pro Met His Ser Asp Gln Pro Arg Asn Ala Val Leu Val			1152
370	375	380	
aca gag gtt ctg aag gtt ggt ttg gtt gtg aag gat tgg gca cag agg Thr Glu Val Leu Lys Val Gly Leu Val Val Lys Asp Trp Ala Gln Arg			1200
385	390	395	400
aat tcg ttg gtg agt gct tca gtt gtt gag aat ggt gtg aga agg ttg Asn Ser Leu Val Ser Ala Ser Val Val Glu Asn Gly Val Arg Arg Leu			1248
405	410	415	
atg gaa aca aag gaa ggt gat gag atg aga cag aga gca gtg agg ctt Met Glu Thr Lys Glu Gly Asp Glu Met Arg Gln Arg Ala Val Arg Leu			1296
420	425	430	
aaa aat gcc atc cat agg tca atg gat gaa ggt gga gtt tct cac atg Lys Asn Ala Ile His Arg Ser Met Asp Glu Gly Val Ser His Met			1344
435	440	445	
gag atg ggt tct ttc att gca cac atc tct aaa Glu Met Gly Ser Phe Ile Ala His Ile Ser Lys			1377
450	455		

<210> 4
 <211> 459
 <212> PRT
 <213> Phaseolus lunatus

<400> 4
 Met Ala Leu Asn Asp Lys Ser Ile Pro His Glu Thr Lys Val Val Val
 1 5 10 15

Leu Leu Ile Pro Phe Pro Ala Gln Gly His Leu Asn Gln Phe Leu His

20

25

30

Leu Ser Arg Leu Ile Val Ala Gln Asn Ile Pro Val His Tyr Val Gly
 35 40 45

Thr Val Thr His Ile Arg Gln Ala Thr Leu Arg Tyr Asn Asn Pro Thr
 50 55 60

Ser Asn Ile His Phe His Ala Phe Gln Val Pro Pro Phe Val Ser Pro
 65 70 75 80

Pro Pro Asn Pro Glu Asp Asp Phe Pro Ser His Leu Ile Pro Ser Phe
 85 90 95

Glu Ala Ser Ala His Leu Arg Glu Pro Val Gly Lys Leu Leu Gln Ser
 100 105 110

Leu Ser Ser Gln Ala Lys Arg Val Val Val Ile Asn Asp Ser Leu Met
 115 120 125

Ala Ser Val Ala Gln Asp Ala Ala Asn Ile Ser Asn Val Glu Asn Tyr
 130 135 140

Thr Phe His Ser Phe Ser Ala Phe Asn Thr Ser Gly Asp Phe Trp Glu
 145 150 155 160

Glu Met Gly Lys Pro Pro Val Gly Asp Phe His Glu Pro Glu Phe Pro
 165 170 175

Ser Leu Glu Gly Cys Ile Ala Ala Gln Phe Lys Gly Phe Arg Thr Ala
 180 185 190

Gln Tyr Glu Phe Arg Lys Phe Asn Asn Gly Asp Ile Tyr Asn Thr Ser
 195 200 205

Arg Val Ile Glu Gly Pro Tyr Val Glu Leu Leu Glu Leu Phe Asn Gly
 210 215 220

Gly Lys Lys Val Trp Ala Leu Gly Pro Phe Asn Pro Leu Ala Val Glu
 225 230 235 240

Lys Lys Asp Ser Ile Gly Phe Arg His Pro Cys Met Glu Trp Leu Asp
 245 250 255

Lys Gln Glu Pro Ser Ser Val Ile Tyr Ile Ser Phe Gly Thr Thr Thr
 260 265 270

Ala Leu Arg Asp Glu Gln Ile Gln Gln Ile Ala Thr Gly Leu Glu Gln
 275 280 285

Ser Lys Gln Lys Phe Ile Trp Val Leu Arg Glu Ala Asp Lys Gly Asp
 290 295 300

Ile Phe Ala Gly Ser Glu Ala Lys Arg Tyr Glu Leu Pro Lys Gly Phe
 305 310 315 320

Glu Glu Arg Val Glu Gly Met Gly Leu Val Val Arg Asp Trp Ala Pro
 325 330 335

Gln Leu Glu Ile Leu Ser His Ser Ser Thr Gly Gly Phe Met Ser His
 340 345 350

Cys Gly Trp Asn Ser Cys Leu Glu Ser Ile Thr Met Gly Val Pro Ile
 355 360 365
 Ala Thr Trp Pro Met His Ser Asp Gln Pro Arg Asn Ala Val Leu Val
 370 375 380
 Thr Glu Val Leu Lys Val Gly Leu Val Val Lys Asp Trp Ala Gln Arg
 385 390 395 400
 Asn Ser Leu Val Ser Ala Ser Val Val Glu Asn Gly Val Arg Arg Leu
 405 410 415
 Met Glu Thr Lys Glu Gly Asp Glu Met Arg Gln Arg Ala Val Arg Leu
 420 425 430
 Lys Asn Ala Ile His Arg Ser Met Asp Glu Gly Gly Val Ser His Met
 435 440 445
 Glu Met Gly Ser Phe Ile Ala His Ile Ser Lys
 450 455

<210> 5
 <211> 503
 <212> PRT
 <213> Phaseolus lunatus

<400> 5
 Met Thr Met Ile Thr Pro Ser Ser Asn Thr Thr His Tyr Arg Glu Ser
 1 5 10 15
 Trp Tyr Ala Cys Arg Tyr Arg Ser Gly Ile Pro Gly Ser Thr His Ala
 20 25 30
 Ser Gly Ser Ile Ala Ser Cys Tyr Pro Asn Ser Pro Met Ala Leu Asn
 35 40 45
 Asp Lys Ser Ile Pro His Glu Thr Lys Val Val Leu Leu Ile Pro
 50 55 60
 Phe Pro Ala Gln Gly His Leu Asn Gln Phe Leu His Leu Ser Arg Leu
 65 70 75 80
 Ile Val Ala Gln Asn Ile Pro Val His Tyr Val Gly Thr Val Thr His
 85 90 95
 Ile Arg Gln Ala Thr Leu Arg Tyr Asn Asn Pro Thr Ser Asn Ile His
 100 105 110
 Phe His Ala Phe Gln Val Pro Pro Phe Val Ser Pro Pro Pro Asn Pro
 115 120 125
 Glu Asp Asp Phe Pro Ser His Leu Ile Pro Ser Phe Glu Ala Ser Ala
 130 135 140
 His Leu Arg Glu Pro Val Gly Lys Leu Leu Gln Ser Leu Ser Ser Gln
 145 150 155 160
 Ala Lys Arg Val Val Ile Asn Asp Ser Leu Met Ala Ser Val Ala
 165 170 175

Gln Asp Ala Ala Asn Ile Ser Asn Val Glu Asn Tyr Thr Phe His Ser
 180 185 190
 Phe Ser Ala Phe Asn Thr Ser Gly Asp Phe Trp Glu Glu Met Gly Lys
 195 200 205
 Pro Pro Val Gly Asp Phe His Glu Pro Glu Phe Pro Ser Leu Glu Gly
 210 215 220
 Cys Ile Ala Ala Gln Phe Lys Gly Phe Arg Thr Ala Gln Tyr Glu Phe
 225 230 235 240
 Arg Lys Phe Asn Asn Gly Asp Ile Tyr Asn Thr Ser Arg Val Ile Glu
 245 250 255
 Gly Pro Tyr Val Glu Leu Leu Glu Leu Phe Asn Gly Gly Lys Lys Val
 260 265 270
 Trp Ala Leu Gly Pro Phe Asn Pro Leu Ala Val Glu Lys Lys Asp Ser
 275 280 285
 Ile Gly Phe Arg His Pro Cys Met Glu Trp Leu Asp Lys Gln Glu Pro
 290 295 300
 Ser Ser Val Ile Tyr Ile Ser Phe Gly Thr Thr Ala Leu Arg Asp
 305 310 315 320
 Glu Gln Ile Gln Gln Ile Ala Thr Gly Leu Glu Gln Ser Lys Gln Lys
 325 330 335
 Phe Ile Trp Val Leu Arg Glu Ala Asp Lys Gly Asp Ile Phe Ala Gly
 340 345 350
 Ser Glu Ala Lys Arg Tyr Glu Leu Pro Lys Gly Phe Glu Glu Arg Val
 355 360 365
 Glu Gly Met Gly Leu Val Val Arg Asp Trp Ala Pro Gln Leu Glu Ile
 370 375 380
 Leu Ser His Ser Ser Thr Gly Gly Phe Met Ser His Cys Gly Trp Asn
 385 390 395 400
 Ser Cys Leu Glu Ser Ile Thr Met Gly Val Pro Ile Ala Thr Trp Pro
 405 410 415
 Met His Ser Asp Gln Pro Arg Asn Ala Val Leu Val Thr Glu Val Leu
 420 425 430
 Lys Val Gly Leu Val Val Lys Asp Trp Ala Gln Arg Asn Ser Leu Val
 435 440 445
 Ser Ala Ser Val Val Glu Asn Gly Val Arg Arg Leu Met Glu Thr Lys
 450 455 460 480
 Glu Gly Asp Glu Met Arg Gln Arg Ala Val Arg Leu Lys Asn Ala Ile
 465 470 475 480
 His Arg Ser Met Asp Glu Gly Gly Val Ser His Met Glu Met Gly Ser
 485 490 495

Phe Ile Ala His Ile Ser Lys
500

<210> 6
<211> 141
<212> DNA
<213> Phaseolus lunatus

<400> 6
aagcttttaa ataagaaata tttttgcta tatataaatac ttgagttataa aatgtatgtt 60
catacttgat ctactacaa ctttaaggata agcagccagt gtcaaagttg aagcatagca 120
tcttgctacc caaattcacc a 141

<210> 7
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR Primer
<400> 7
gaagcatagc atcttgctac c 21

<210> 8
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR Primer
<400> 8
caacagaagc ttttatttttg g 21

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR Primer
<400> 9
atggctttga atgacaaaag c 21

<210> 10
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:PCR primer

<400> 10

ctat tagat atgtgtgcaaa tg

22

<210> 11

<211> 1365

<212> DNA

<213> Phaseolus vulgaris

<220>

<221> CDS

<222> (1)...(1365)

<400> 11

atg	gct	ttg	aat	gat	gaa	acc	aaa	gtg	gtg	gtg	ctt	ttg	cta	cct	ttc	48
Met	Ala	Leu	Asn	Asp	Glu	Thr	Lys	Val	Val	Val	Leu	Leu	Leu	Pro	Phe	
1		5						10						15		

cct	gta	caa	ggt	cac	ctt	aac	ccg	ttt	ctg	cag	cta	tcc	cac	cta	atc	96
Pro	Val	Gln	Gly	His	Leu	Asn	Pro	Phe	Leu	Gln	Leu	Ser	His	Ile		
		20						25				30				

gcg	gca	caa	aac	ata	gca	gtg	cat	tat	gtt	ggc	act	gtc	aca	cac	att	144
Ala	Ala	Gln	Asn	Ile	Ala	Val	His	Tyr	Val	Gly	Thr	Val	Thr	His	Ile	
		35				40			45							

cgc	caa	gca	aaa	ctt	cga	tac	cac	aac	gct	act	tca	aac	atc	cat	ttc	192
Arg	Gln	Ala	Lys	Leu	Arg	Tyr	His	Asn	Ala	Thr	Ser	Asn	Ile	His	Phe	
		50			55				60							

cat	gcc	ttt	gaa	gtt	cca	ccc	tat	gtt	tct	cct	ccc	aat	cca	gaa	240
His	Ala	Phe	Glu	Val	Pro	Pro	Tyr	Val	Ser	Pro	Pro	Pro	Asn	Pro	Glu
		65			70			75					80		

gac	gat	ttc	cca	tct	cat	ctc	att	cct	tcc	ttt	gag	gcc	tct	gca	cac	288
Asp	Asp	Phe	Pro	Ser	His	Leu	Ile	Pro	Ser	Phe	Glu	Ala	Ser	Ala	His	
					85			90				95				

ctt	cgg	gag	cct	gtg	ggg	aaa	ctg	ctt	caa	tcc	ctt	tcg	tca	caa	gcc	336
Leu	Arg	Glu	Pro	Val	Gly	Lys	Leu	Leu	Gln	Ser	Leu	Ser	Ser	Gln	Ala	
						100		105				110				

aaa	agg	gtc	gta	ctc	atc	aat	gac	tcc	ctc	atg	gca	tcc	gtg	gca	caa	384
Lys	Arg	Val	Val	Leu	Ile	Asn	Asp	Ser	Leu	Met	Ala	Ser	Val	Ala	Gln	
					115			120			125					

gat	gct	gca	aac	ttc	tca	aat	gtt	gaa	aga	tac	tgt	ttt	caa	gtc	ttc	432
Asp	Ala	Ala	Asn	Phe	Ser	Asn	Val	Glu	Arg	Tyr	Cys	Phe	Gln	Val	Phe	
					130			135			140					

tct	gcc	ctt	aat	acc	gcc	ggt	gat	ttt	tgg	gaa	caa	atg	gga	aag	ccc	480
Ser	Ala	Leu	Asn	Thr	Ala	Gly	Asp	Phe	Trp	Glu	Gln	Met	Gly	Lys	Pro	
					145			150			155		160			

cct	ctt	gca	gat	ttc	cat	ttc	cca	gat	att	cct	tct	ctc	caa	gga	tgc	528
Pro	Leu	Ala	Asp	Phe	His	Phe	Pro	Asp	Ile	Pro	Ser	Leu	Gln	Gly	Cys	
					165			170			175					

atc	tca	gcc	cag	ttc	acg	gat	ttt	ctt	act	gca	cag	aat	gaa	ttc	cgc	576
Ile	Ser	Ala	Gln	Phe	Thr	Asp	Phe	Leu	Thr	Ala	Gln	Asn	Glu	Phe	Arg	
					180			185			190					

aaa ttc aac aat ggc gat att tac aac acc agc agg gtg att gag ggt	624
Lys Phe Asn Asn Gly Asp Ile Tyr Asn Thr Ser Arg Val Ile Glu Gly	
195 200 205	
cct tac gtt gag ttg ctg gag cgt ttc aat ggc ggc aag gag gtt tgg	672
Pro Tyr Val Glu Leu Leu Glu Arg Phe Asn Gly Gly Lys Glu Val Trp	
210 215 220	
gca ctg ggg cca ttc acc cct tta gcc gtt gag aag aaa gat tcg ata	720
Ala Leu Gly Pro Phe Thr Pro Leu Ala Val Glu Lys Lys Asp Ser Ile	
225 230 235 240	
gga ttt agt cac cca tgc atg gag tgg ctt gat aaa caa gag cca agt	768
Gly Phe Ser His Pro Cys Met Glu Trp Leu Asp Lys Gln Glu Pro Ser	
245 250 255	
tca gtg ata tat gta tcc ttc ggg acc acc aca gct ttg aga gat gaa	816
Ser Val Ile Tyr Val Ser Phe Gly Thr Thr Ala Leu Arg Asp Glu	
260 265 270	
caa atc caa gag cta gca act ggg ttg gaa cag agc aag cag aag ttc	864
Gln Ile Gln Glu Leu Ala Thr Gly Leu Glu Gln Ser Lys Gln Lys Phe	
275 280 285	
atc tgg gtg ctg aga gat gct gat aaa ggt gat atc ttt gac gga agt	912
Ile Trp Val Leu Arg Asp Ala Asp Lys Gly Asp Ile Phe Asp Gly Ser	
290 295 300	
gaa gca aaa agg tat gaa ctt cca gag ggt ttt gag gag aga gtg gaa	960
Glu Ala Lys Arg Tyr Glu Leu Pro Glu Gly Phe Glu Glu Arg Val Glu	
305 310 315 320	
gga atg ggg ctg gtt gtg agg gat tgg gca ccc caa atg gaa att ctg	1008
Gly Met Gly Leu Val Val Arg Asp Trp Ala Pro Gln Met Glu Ile Leu	
325 330 335	
agc cac agt tca aca ggg ggg ttc atg agc cat tgt gga tgg aac tcg	1056
Ser His Ser Thr Gly Gly Phe Met Ser His Cys Gly Trp Asn Ser	
340 345 350	
tgc ttg gag agc tta acc agg ggg gtg cca atg gct aca tgg gcc atg	1104
Cys Leu Glu Ser Leu Thr Arg Gly Val Pro Met Ala Thr Trp Ala Met	
355 360 365	
cac tct gac cag cca aga aat gca gtt ttg gtg aca gat gtt ctg aag	1152
His Ser Asp Gln Pro Arg Asn Ala Val Leu Val Thr Asp Val Leu Lys	
370 375 380	
gtt ggt ttg att gtg aag gat tgg gaa cag aga aaa tcg ttg gtg agt	1200
Val Gly Leu Ile Val Lys Asp Trp Glu Gln Arg Lys Ser Leu Val Ser	
385 390 395 400	
gct tca gtt att gag aat gct gtg aga agg ttg atg gaa aca aag gaa	1248
Ala Ser Val Ile Glu Asn Ala Val Arg Arg Leu Met Glu Thr Lys Glu	
405 410 415	
ggt gat gag atc aga aag aga gca gtg aag ctt aaa gat gag atc cat	1296
Gly Asp Glu Ile Arg Lys Arg Ala Val Lys Leu Lys Asp Glu Ile His	
420 425 430	

agg tcc atg gat gaa ggt gga gtt tct cgc atg gag atg gct tct ttc 1344
 Arg Ser Met Asp Glu Gly Gly Val Ser Arg Met Glu Met Ala Ser Phe
 435 440 445

 att gca cac atc tct aga tag 1365
 Ile Ala His Ile Ser Arg
 450 455

 <210> 12
 <211> 454
 <212> PRT
 <213> Phaseolus vulgaris

 <400> 12
 Met Ala Leu Asn Asp Glu Thr Lys Val Val Val Leu Leu Leu Pro Phe
 1 5 10 15

 Pro Val Gln Gly His Leu Asn Pro Phe Leu Gln Leu Ser His Leu Ile
 20 25 30

 Ala Ala Gln Asn Ile Ala Val His Tyr Val Gly Thr Val Thr His Ile
 35 40 45

 Arg Gln Ala Lys Leu Arg Tyr His Asn Ala Thr Ser Asn Ile His Phe
 50 55 60

 His Ala Phe Glu Val Pro Pro Tyr Val Ser Pro Pro Pro Asn Pro Glu
 65 70 75 80

 Asp Asp Phe Pro Ser His Leu Ile Pro Ser Phe Glu Ala Ser Ala His
 85 90 95

 Leu Arg Glu Pro Val Gly Lys Leu Leu Gln Ser Leu Ser Ser Gln Ala
 100 105 110

 Lys Arg Val Val Leu Ile Asn Asp Ser Leu Met Ala Ser Val Ala Gln
 115 120 125

 Asp Ala Ala Asn Phe Ser Asn Val Glu Arg Tyr Cys Phe Gln Val Phe
 130 135 140

 Ser Ala Leu Asn Thr Ala Gly Asp Phe Trp Glu Gln Met Gly Lys Pro
 145 150 155 160

 Pro Leu Ala Asp Phe His Phe Pro Asp Ile Pro Ser Leu Gln Gly Cys
 165 170 175

 Ile Ser Ala Gln Phe Thr Asp Phe Leu Thr Ala Gln Asn Glu Phe Arg
 180 185 190

 Lys Phe Asn Asn Gly Asp Ile Tyr Asn Thr Ser Arg Val Ile Glu Gly
 195 200 205

 Pro Tyr Val Glu Leu Leu Glu Arg Phe Asn Gly Gly Lys Glu Val Trp
 210 215 220

 Ala Leu Gly Pro Phe Thr Pro Leu Ala Val Glu Lys Lys Asp Ser Ile
 225 230 235 240

 Gly Phe Ser His Pro Cys Met Glu Trp Leu Asp Lys Gln Glu Pro Ser

245	250	255
Ser Val Ile Tyr Val Ser Phe Gly Thr Thr Thr Ala Leu Arg Asp Glu		
260	265	270
Gln Ile Gln Glu Leu Ala Thr Gly Leu Glu Gln Ser Lys Gln Lys Phe		
275	280	285
Ile Trp Val Leu Arg Asp Ala Asp Lys Gly Asp Ile Phe Asp Gly Ser		
290	295	300
Glu Ala Lys Arg Tyr Glu Leu Pro Glu Gly Phe Glu Glu Arg Val Glu		
305	310	315
Gly Met Gly Leu Val Val Arg Asp Trp Ala Pro Gln Met Glu Ile Leu		
325	330	335
Ser His Ser Ser Thr Gly Gly Phe Met Ser His Cys Gly Trp Asn Ser		
340	345	350
Cys Leu Glu Ser Leu Thr Arg Gly Val Pro Met Ala Thr Trp Ala Met		
355	360	365
His Ser Asp Gln Pro Arg Asn Ala Val Leu Val Thr Asp Val Leu Lys		
370	375	380
Val Gly Leu Ile Val Lys Asp Trp Glu Gln Arg Lys Ser Leu Val Ser		
385	390	395
Ala Ser Val Ile Glu Asn Ala Val Arg Arg Leu Met Glu Thr Lys Glu		
405	410	415
Gly Asp Glu Ile Arg Lys Arg Ala Val Lys Leu Lys Asp Glu Ile His		
420	425	430
Arg Ser Met Asp Glu Gly Gly Val Ser Arg Met Glu Met Ala Ser Phe		
435	440	445
Ile Ala His Ile Ser Arg		
450		

<210> 13
 <211> 26
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: PCR Primer

<400> 13
 catggagatg ggttcttca ttgcac 26

<210> 14
 <211> 24
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: PCR Primer

<400> 14
caacaactga agcactcacc aacg 24

<210> 15
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR Primer

<400> 15
ccaaagtgcga caatggcttt gaatgatg 28

<210> 16
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: PCR Primer

<400> 16
gctatgcggc cgcctaaatg gtatgac 27

1

11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/27759

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/82, 5/04, 15/29, 15/05, 15/09; A01H 4/00, 5/10
 US CL :536/23.6, 23.1; 435/69.1, 468, 410, 419, 420; 800/278

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.6, 23.1; 435/69.1, 468, 410, 419, 420; 800/278

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, AGRICOLA, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HOVARTH et al. Identification of an Immediate-Early Salicylic Acid-Inducible Tobacco Gene and Characterization of Induction by Other Compounds. Plant Molecular Biology. 1996, Vol. 31, No. 5, pages 1061-1072, see entire document.	1-24

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:		
"A" document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 MARCH 1999

Date of mailing of the international search report

08 APR 1999

Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

 OUSAMA M-FAIZ ZAGHMOUT

Telephone No. (703) 308-0196